Role of Vascular Endothelial Growth Factor (VEGF) in granulosa cell function: Involvement of heterotrimeric G-protein signalling pathways.

By

Lynsey Kerr Doyle

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Declaration

I hereby declare that this thesis has been composed by myself and has not been submitted for any other degree in Edinburgh or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge all assistance given to me during the design and execution of the experiments contained in this thesis and its preparation.

Lynsey Kerr Doyle

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Abstract

Vascular Endothelial Growth Factor (VEGF) has been shown to be an absolute requirement for ovarian follicle development. Although VEGF is commonly regarded primarily as an angiogenic factor, granulosa cells are a major site of VEGF synthesis in the follicle and they express VEGF receptors (VEGFR1 and VEGFR2). Further, the development of the dominant follicle is characterised by a substantial increase in granulosa cell expression of VEGF and its receptors. In spite of this, potential non-angiogenic effects of VEGF in these follicles have not been elucidated. The objective of the three studies described in this thesis was to use an in vitro bovine granulosa cell model to investigate the roles of VEGF during development of the dominant follicle. In addition, in light of evidence in other cell types, potential interactions between VEGF signalling and heterotrimeric protein signalling in these follicles were also investigated.

In the first study, granulosa cells were obtained from healthy follicles with diameters of 4 to 8 mm (corresponding to just before the selection of a dominant follicle during a follicular wave) or 9 to 14 mm (encompassing all developmental stages of a dominant follicle) and exposed to a range of VEGF concentrations (1 to 100 ng/ml) encompassing concentrations found naturally in bovine dominant follicles. VEGF at 1 ng/ml, but not at higher concentrations ($P > 0.1$), induced significant proliferation of bovine granulosa cells from 4 to 8 mm follicles ($P = 0.024$) and increased the proliferative response of these cells to FSH ($P = 0.045$). VEGF also induced a dose-dependent increase in ERK1/2 activation by granulosa cells from 4 to 8 mm follicles ($P < 0.03$) but did not have any effect on expression of the steroidogenic enzyme, *CYP11A1*, by these cells ($P > 0.1$). VEGF, at a dose of 1 ng/ml ($P = 0.003$), but not at higher doses ($P > 0.1$), induced an increase in *COX-2* expression by granulosa cells from 9 to 14 mm follicles. In addition, LH stimulation of both ERK phosphorylation ($P < 0.05$) and *COX-2* expression ($P < 0.05$) in granulosa cells from 9 to 14 mm follicles were prevented ($P > 0.1$) by specific inhibition of VEGFR2, indicating that VEGF may mediate *COX-2* responses to LH in these cells.

The second study sought to examine the expression of heterotrimeric G-protein α subunits and PLCβ isoforms by real-time PCR and westen blotting in bovine granulosa cells throughout follicle development to identify specific molecular components of heterotrimeric G-protein pathways that may functionally interact with intracellular VEGF signals. Results showed that *GNAS*, *GNA11* and *GNAI2* were all expressed at significantly ($P < 0.05$) higher levels in granulosa cells of pre-ovulatory-size follicles (10.0 to 13.9 mm) than in cells from smaller follicles (2.0 to 5.9 mm and 6.0 to 9.9 mm). In addition, all PLCB isoforms except *PLCB2* were expressed in bovine granulosa cells with *PLCB3* being more abundant than *PLCB1* and *-4*. Levels of *PLCB3* in granulosa cells from pre-ovulatory-size follicles were much higher (>16-fold; $P < 0.005$) than in smaller follicles. Immunocytochemical analysis revealed that *PLCB3* was located primarily in the cytoplasm, whereas *PLCB1* was distributed primarily in the nucleus. These results identified Gs, Gq/11, Gi2 and PLCβ3 as candidates for cross-talk between VEGF and heterotrimeric G-protein signalling during the development of the dominant follicle.

The potential involvement of these molecules on VEGF-induced responses in granulosa cells from 9-14 mm follicles was investigated in the third study by determining the effects of specific inhibitors of Gi (pertussis toxin, PTX) or Gq/11 (YM-25489) or PLCB3 siRNAs on VEGF-induced p-ERK. Results showed a 2.3–fold mean increase in p-ERK in response to VEGF in the absence of G protein inhibitors ($P < 0.0001$) but a VEGF response that was completely or partially abolished, respectively, in the presence of PTX ($P > 0.8$) or YM-25489 (1.6-fold mean increase relative to untreated controls; $P = 0.039$). LH induced a 1.6–fold increase in p-ERK1/2 ($P < 0.02$) and this response was prevented by pre-incubation with PTX ($P > 0.4$) or YM-25489 ($P > 0.5$). In contrast, similar EGF-induced phosphorylation of ERK (about 5-fold relative to controls) occurred in the absence ($P < 0.003$) or presence of PTX ($P < 0.003$) or YM-25489 ($P < 0.003$). Transfection of granulosa cells with 3 siRNAs targeting PLCB3 that had been previously validated by western blotting and immunocytochemistry had no effect ($P = > 0.7$) on phosphorylation of ERK in response to VEGF, LH or EGF in granulosa cells.

In conclusion, taken together, these results suggest novel roles of VEGF in stimulating granulosa cell proliferation and expression of *COX-2* in bovine dominant follicles and implicate VEGF in synergising and/or mediating the effects of gonadotrophins in these cells. In addition, these results indicate a requirement for Gi2 and Gq/11 in VEGF activation of ERK1/2 and induction of the above responses in granulosa cells.

Publications

APPENDIX 1: Doyle,L., Hogg,C., Watson, E., Donadeu,X.(2008). Seasonal effects on the response of ovarian follicles to IGF-I in mares. Reproduction. Advanced publication, first posted online on 7th August 2008.

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Conference Abstracts:

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List of Abbreviations

AC	adenylyl cyclase
ADAMTS	a disintigrin and metalloproteinase with thrombospondin motif
ADP	adenosine diphosphate
AF	annulus fibrosus
Akt	protein kinase B
AR	amphiregulin
ATP	adenosine triphosphate
GalTase	β-1, 4, galactosyltransferase
bFGF	basic fibroblast growth factor
BOEC	bovine oviductal epithelial cells
BSA	bovine serum albumin
BTC	beta-cellulin
cAMP	cyclic adenosine monophosphate
CAV	caveolin
CCl_4	carbon tetrachloride
cDNA	complementary deoxyribonucleic acid
COC	cumulus oocyte complex
COX	cyclooxygenase
CREB	cAMP response element binding
DAG	diacylglycerol
DTT	dithiothreitol
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPI	epiregulin
ERK	extracellular regulated kinase
ET	endothelin
FGF	fibroblast growth factor
FICT	fluorescein isothiocyanate
Flk	fetal liver kinase

Flt	fms-like tyrosine kinase
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GDF	growth differentiation factor
GDP	guanosine diphosphate
GFP	green fluorescent protein
GH	growth hormone
GnRH	gonadotrophin releasing hormone
GPCR	G protein coupled receptor
Grb2	growth factor receptor bound protein 2 adaptor protein
GTP	guanosine-5’-triphosphate
hCG	human chorionic gonadotrophin
HDFC	human dental follicle cell
HGF	hepatocyte growth factor
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
IFGR	insulin-like growth factor receptor
Ig	immunoglobulin
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IP3	inositol triphosphate
ITS	insulin, transferring and sodium selenite
IVM	in vitro maturation
JNK	jun N-terminal kinase
kDa	kilodalton
KDR	kinase insert domain receptor
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LPA	lysophosphatidic acid
mA	milliamps
mg	milligram
ml	millilitre

MAPK	mitogen activated protein kinase
MEK	map-erk kinase
mm	milimeter
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
mRNA	messenger ribonucleic acid
ng	nanogram
nM	nanomolar
NGS	normal goat serum
NHEK	normal human epidermal keratinocyte
nM	nanomolar
NP	nucleus pulposus
NRP	neuropilan
P1GF	placental growth factor
PAPP-A	pregnancy associated plasma protein A
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
p-ERK	phosphorylated extracellular regulated kinase
PFA	paraformaldehyde
PG	prostaglandin
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositiol biphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTEN	phosphatase and tensis homologue
PTX	pertussis toxin
qPCR	quantitative polymerase chain reaction
RACK	receptors for activated protein kinase C
RGS	regulator of G protein signalling

rh	recombinant human
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SAPK	stress activated protein kinase
P450scc	cytochrome P450side chain cleavage
SDF	stromal derived factor
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
Sos	Son of sevenless
StAR	steroidogenic acute regulatory protein
TBS	tris buffered saline
Tfsv	Trimeresurus flavoviridis snake venom
TGF	transforming growth factor
TNF	tumour necrosis factor
µg	microgram
µl	microlitre
µM	micromolar
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VPF	vascular permeability factor
ZP	zona pellucida

CHAPTER 1:

Literature Review

1.1 Folliculogenesis

Development of a preovulatory follicle in mammalian species is the first milestone towards successful reproduction. The ovarian follicle plays a fundamental role in reproductive outcome and as such it is imperative that the mechanisms of follicle development are fully understood in order to improve and control reproductive function in animals and humans alike. The lack of availability of human ovarian tissue severely restricts research in the field of human reproduction, therefore other species are commonly used as models for the human (Reviewed by Campbell, Souza, Gong et al. 2003). Cattle have been proposed as physiologically relevant models for the study of the basic mechanisms of folliculogenesis (Malhi, Adams, & Singh 2005; Adams & Pierson 1995). The large size and relative ease by which follicles in cattle can be monitored, together with the similarities that have been demonstrated between follicle dynamics in the cow and human have offered justification for this model.

1.1.1 Stages of follicle development

Folliculogenesis occurs within the cortex of the ovary, where follicles develop to increasingly higher levels of organisation through proliferation and differentiation of both the oocyte and associated follicular somatic cells. Folliculogenesis involves the sequential development of a pool of primordial follicles into primary, secondary and tertiary (Graafian or antral) follicles from which a preovulatory follicle may eventually arise (Figure 1.1, Table 1.1).

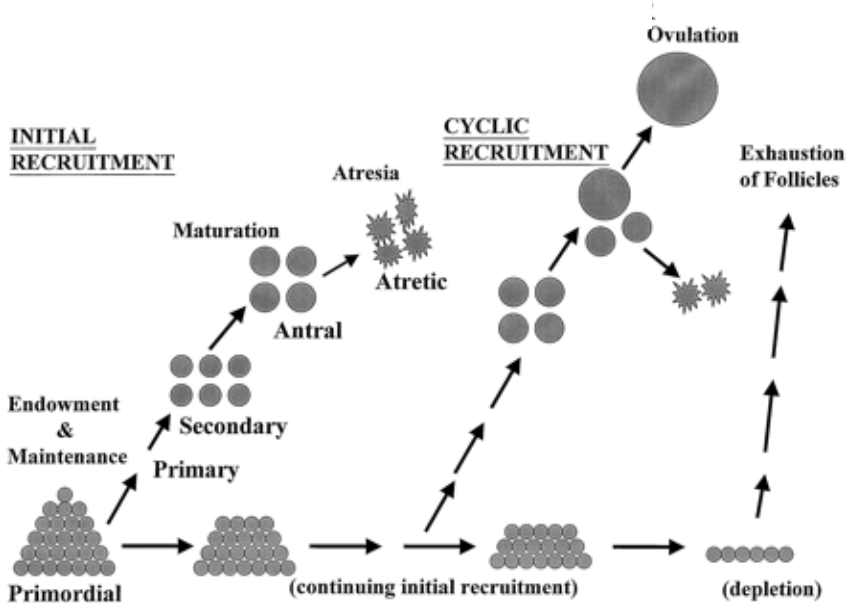


Figure 1.1. Follicle development in mammalian species. There is a fixed population of primordial follicles at birth, the majority of which are maintained in a resting state. Growth of some of these follicles is initiated at or shortly after birth and continues throughout reproductive life. Follicles develop through primary and secondary stages of development prior to the formation of an antral cavity. The majority of antral follicles undergo atresia. However under appropriate gonadotrophin stimulation, some antral follicles develop to the preovulatory stage. Eventually the pool of resting follicles becomes exhausted. Taken from (McGee & Hsueh 2000).

1.1.1.1 Primordial Follicles

Primordial follicles form when primary oocytes become surrounded by a single layer of flattened pre-granulosa cells (Braw-Tal & Yossefi 1997). In rodents, formation of primordial follicles occurs in a synchronous manner during the first few days after birth. In contrast, formation of primordial follicles in domestic species and humans occurs during foetal life. Primordial follicles are thought to be initially maintained in a resting state. Resting follicles are likely to be under the influence of both local and systemic inhibitory factors and only when these inhibitory effects decline or stimulatory factors are released will follicle growth be initiated (McGee & Hsueh 2000). Cattle have a reserve of approximately 150,000 primordial follicles in the ovary at the time of birth (Erickson 1966; Ireland 1987). This population

subsequently falls to approximately 3000 follicles by 15 to 20 years of age (Erickson 1966), reviewed by (Webb, Gong, Law et al. 1992). Once a primordial follicle enters the pool of growing follicles (a process that occurs continuously throughout life), it will develop through distinct morphological stages until ovulation or until it degenerates by atresia, which is the fate of the majority (>99%) of follicles (Ireland 1987).

1.1.1.2 Primary and secondary follicles

Development through primary and secondary stages of follicle growth involves the progressive acquisition of several layers of cuboidal granulosa cells, see table 1.1. Secondary follicles enlarge and recruited stromal cells close to the basal lamina of the follicle differentiate to form the thecal layer (composed of theca interna and externa). In rodent follicles, the thecal layer is present as early as the late primary to early secondary stage (Fortune 2003); this is in contrast with bovine (Braw-Tal & Yossefi 1997), ovine (Lundy, Smith, O'Connell et al. 1999) and primates (Gougeon 1996), in which the thecal layer can not be identified until the mid to late secondary stage.

1.1.1.3 Antral (tertiary) Follicles

Bovine preantral follicles progress to the early antral stage upon reaching a diameter of 115-280μm (Gordon 2003), at which point they begin to form an antrum. The formation of the antrum results from the formation of small fluid filled cavities (lacunae) within the granulosa cell layer that eventually coalesce (Van Voorhis 1999). Follicle development up to early antral stage is not dependent on gonadotrophic influences (Scaramuzzi, Adams, Baird et al. 1993) and represents a relatively slow stage of growth, for example it takes approximately 30 days for a bovine follicle to grow from 300 μm to 5mm (Lussier, Matton, & Dufour 1987). Subsequent follicle growth up to a preovulatory diameter of 15 to 20 mm is rapid and occurs over the following 4 to 6 days (Evans & Canty 2004). Gonadotrophic support for continued follicle development becomes absolutely essential after the early antral

stage (after follicles reach 2mm in diameter in cattle) (Campbell, Scaramuzzi, & Webb 1995; Campbell, Souza, Gong et al. 2003).

Progression through the antral stages of follicle development in large domestic species can easily be monitored in real-time by ultrasonography (Reviewed by Ginther 1995), therefore relatively much more is known about the physiology of these latter stages of follicle development compared to earlier stages.

Table 1.1. Chacteristics of bovine follicle developmental stages.
Taken from (Braw-Tal & Yossefi 1997).

Follicle	Number of Granulosa Cell Layers	Granulosa Cell Numbers	Follicle Diameter, (range,µm)	Oocyte Diameter (µm ,mean, ±S.E.M)	Presence of Zona Pellucida	Presence of Theca
Primordial	1	<10 (flattened)	<40	29.7 ± 0.3	-	-
Primary	1-1.5	10-40 cuboidal	40-80	31.1 ± 0.4	-	-
Small Secondary	2-3	41-100	81-130	49.5 ± 2.4	-	-
Large Secondary	4-6	101-250	131-250	68.6 ± 2.8	+	+
Small Antral	>6	>250	250-500	92.9 ± 4.5	++	++

1.1.2 Follicle Waves

Antral follicles in the mammalian ovary were first described by Dutch anatomist, Reigner de Graff (1641-1673). De Graff demonstrated that there were a number of follicles present in the ovary at any one time; however, the relationships among these follicles remained elusive for over 300 years. In 1928 McNutt concluded that large follicles in non-pregnant cattle follow cyclical changes and normally grow to their largest size during the latter half of the 21 day cycle (McNutt 1928). Initially, investigators believed that follicular development was a continuous process that occurred independently of phases of the oestrous cycle but in 1960, Rajakoski proposed that cattle have two follicular waves during a single estrous cycle (Rajakoski 1960). For three decades, this suggestion was controversial as there were numerous studies that either supported (Pierson & Ginther 1987; Hackett & Hafs

1969; Ireland, Coulson, & Murphree 1979; Swanson, Hafs, & Morrow 1972) or refuted (Donaldson & Hansel 1968; Dufour, Whitmore, Ginther et al. 1972; Ireland & Roche 1983; Matton, Adelakoun, Couture et al. 1981; Priedkalns, Weber, & Zemjanis 1968; Spicer & Echternkamp 1986) that claim. It was not until the introduction of ultrasonography in veterinary science that the knowledge barrier was broken and progress towards the current understanding of follicular dynamics was initiated. The first full description of bovine ovarian follicular dynamics throughout a complete oestrous cycle was done using ultrasonographic analysis (Sirois & Fortune 1988). Ultrasonography is a non-invasive tool used to monitor individual follicles on consecutive days in the same animal as they grow and regress, allowing patterns of follicle development to be determined with relative precision (Ginther, Knopf & Kastelic 1989).

The term follicle wave refers to the growth of follicles that emerge as a cohort and can be detected by standard ultrasonographic techniques, i.e., follicles $\geq$ 2 mm (Mihm & Austin 2002). Typically, 2 to 3 waves per oestrous cycle are observed in cattle (Reviewed in Adams, Jaiswal, Singh et al. 2008) but there is a propensity for specific breeds to display a characteristic number of waves. For example lactating Holstein cattle tend to have two follicle waves per cycle (Taylor & Rajamahendran 1991; Townson, Tsang, Butler et al. 2002) whereas beef and dairy heifers tend to have 2 or 3 waves per cycle (Ginther, Knopf, & Kastelic 1989; Savio, Keenan, Boland et al. 1988; Sirois & Fortune 1988). Tropical *Bos Indicus* cattle have been observed to have as many as 4 waves per cycle (Bo, Baruselli, & Martonez 2003).

Approximately every 10 days during an oestrous cycle, a follicle wave emerges under stimulation by a preceding surge in circulating FSH concentrations (Adams, Matteri, Kastelic et al. 1992; Ginther, Beg, Gastal et al. 2005; Sunderland, Crowe, Boland et al. 1994). Each follicular wave is characterised by the synchronous growth of a group (cohort) of follicles. Recruitment of follicles into the cohort occurs during a window of time of 2 days in cattle (Driancourt 2001). The number of follicles recruited into the cohort differs between species, but ranges from 5 to 10 in cattle

(Lussier, Matton & Dufour 1987). Initially all follicles in a cohort enter a common growth phase during which they grow at an approximately similar rate (Figure 1.2).

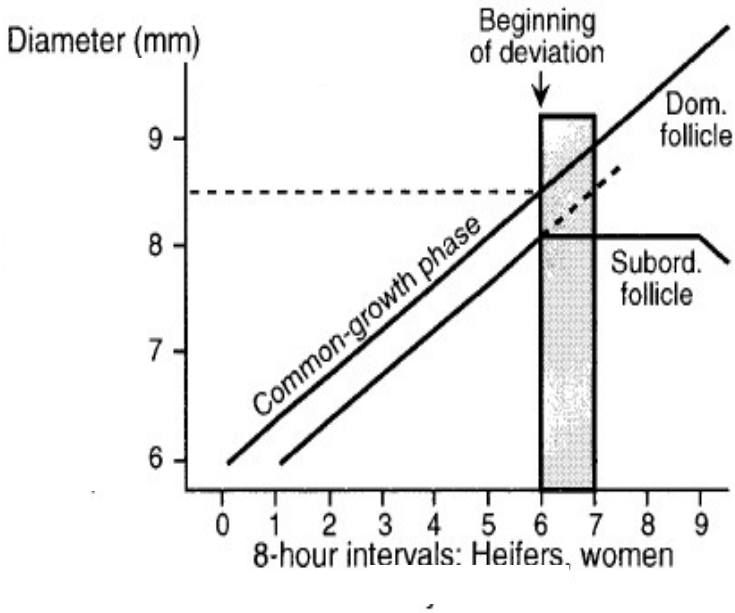


Figure 1.2. Schematic depiction of diameter profiles of the dominant and largest subordinate follicles of a wave in cattle and women. Adapted from (Ginther, Beg, Bergfelt et al. 2001).

By the end of the common growth phase only the largest (future dominant) follicle is able to maintain its growth rate, whilst the remaining (subordinate) follicles slow down or cease their growth before starting to regress (Reviewed by Ginther, Bergfelt, Beg et al. 2001a). The two largest follicles of a wave begin to deviate in diameter when the largest follicle reaches a diameter of 8.5 mm in cattle (Ginther, Beg, Donadeu et al. 2003). The selected follicle will either ovulate (dominant ovulatory follicle) or become atretic (dominant non-ovulatory follicle) depending on whether it develops in the presence (follicular phase) or absence (luteal phase) of high progesterone levels in circulation (Reviewed by Bao & Garverick 1998; Kastelic, Knopf, & Ginther 1990).

The future dominant (normally the largest) follicle has been shown to emerge 6-10 hours before the largest future subordinate follicle (Ginther, Kot, Kulick et al. 1997a; Kulick, Kot, Wiltbank et al. 1999). This provides the future dominant follicle with a size advantage of about 0.5 mm relative to the second largest follicle (future largest subordinate) (Ginther, Beg, Donadeu, et al. 2003). Throughout the common growth

phase, all follicles are capable of becoming dominant as has been shown using ablation experiments. For example, when the largest follicle was ablated at the end of the common growth phase in both heifers and mares, the second largest follicle was capable of becoming dominant (Ginther, Beg, Donadeu et al 2003). Similarly a study in cattle found that when one, two or three of the largest follicles were ablated at the end of the common growth phase, of the remaining follicles the first to reach a diameter of 7mm became dominant (Ginther, Bergfelt, Beg, & Kot 2001a). Despite the largest follicle having obvious morphological dominance as indicated by its significantly larger size compared to others in the cohort during deviation, it remains difficult to pin-point the exact time that follicle selection begins because prior to diameter deviation distinct intrafollicular biochemical events occur that may prepare the largest follicle to become dominant (biochemical dominance) (Fortune, Rivera, & Yang 2004).

1.1.3 Regulation of follicle selection and development of the dominant Follicle

Follicle selection and the subsequent development of the dominant follicle to preovulatory stage has been extensively studied, particularly in large monovulatory species such as the cow and mare (Reviewed by Ginther 2000). Both systemic and local regulatory mechanisms have been shown to control this process.

1.1.3.1 Systemic regulatory mechanisms

Follicle Stimulating Hormone (FSH). A two-way coupling between FSH concentrations and the follicles of a wave has been shown whereby FSH stimulates follicle growth whilst the growing follicles exert an inhibitory effect on the concentrations of the FSH surge (Figure 1.3) (Ginther, Bergfelt, Kulick et al. 1999; Ginther, Bergfelt, Kulick et al. 2000b). Follicular requirements for FSH have been demonstrated in functional studies, whereby inhibition of FSH resulted in an inhibition of follicle emergence. This effect could be prevented by administration of exogenous FSH (Reviewed in Mihm & Bleach 2003). The concentrations of the FSH

surge begin to decline when the largest follicle reaches 4 mm and this is due to the FSH-suppressing activities of inhibin secreted by all growing follicles (Bleach, Glencross, Feist et al. 2001; Gibbons, Wiltbank, & Ginther 1997; Mihm, Good, Ireland et al. 1997; Sunderland, Knight, Boland et al. 1996). The role of inhibin-A in the feedback regulation of FSH concentration has been shown in a number of studies in which immunoneutralisation of inhibin-A resulted in significantly increased levels of plasma FSH (Glencross, Bleach, McLeod et al. 1992; Kaneko, Nakanishi, Akagi et al. 1995; Kaneko, Taya, Watanabe et al. 1997). Further support was provided by studies in heifers whereby administration of inhibin resulted in a significant reduction in plasma FSH levels (Beard, Castillo, McLeod et al. 1990; Beard, Savva, Glencross et al. 1989; Turzillo & Fortune 1993).

Just before the beginning of deviation, the future dominant follicle begins to produce increased oestradiol concentrations and both inhibin and oestradiol contribute to the continuous suppression of FSH throughout the development of the dominant follicle (Ginther, Bergfelt, Kulick et al. 2000a). It has been concluded that the combined effects of follicular inhibin and oestradiol at the beginning of deviation drive the decline in FSH concentration to levels below the requirement of the smaller follicles thus preventing their continued growth (Figure 1.3) (Ginther, Bergfelt, Kulick, et al. 2000a). The largest follicle, however, acquires the ability to more efficiently utilise the reduced concentrations of FSH at the beginning of deviation so it can continue growing (Ginther 2000; Ginther, Beg, Bergfelt et al 2001), as described below. The decline in FSH concentration has been shown to be critical for the selection of the dominant follicle in functional studies that involved artificial manipulation of FSH concentrations in cattle and this forms the basis for treatments to induce superovulation in domestic species (Adams, Kot, Smith et al. 1993; Hampton, Bader, Lamberson et al. 2004; Mihm, Good, Ireland et al. 1997).

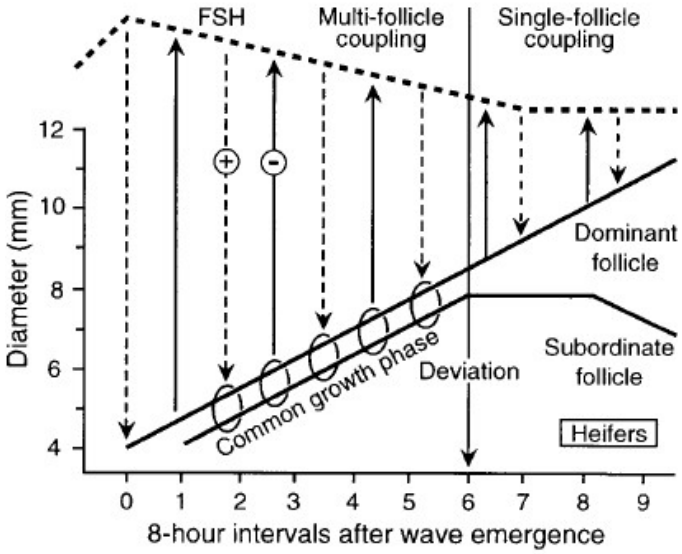


Figure 1.3. FSH-Follicle relationship during a follicular wave in bovine. Amended from (Ginther, Beg, Bergfelt, et al. 2001). Initially all follicles of a wave utilise circulating FSH for growth and at the same time contribute to the FSH decline. At the beginning of deviation the dominant follicle becomes soley responsible for the continuous supression of FSH. At that time circulating FSH levels are sufficient to maintain growth of the largest (dominant) follicle but are too low to support smaller (subordinate) follicles.

Luteinising Hormone (LH). A transition from FSH to LH-dependency by the dominant follicle has been proposed as a potential mechanism in the continued development of the dominant follicle (Reviewed in Mihm & Evans 2008). In cattle, there is a transient increase in intrafollicular LH concentrations 24-32 hours before the beginning of deviation which remain elevated until 24 hours post deviation (Ginther, Bergfelt, Kulick et al. 1998; Kulick, Kot, Wiltbank et al. 1999). In addition, experimental suppression of LH results in a failure of the dominant follicle to develop in both cows (Gong, Campbell, Bramley et al. 1996) and horses (Gastal, Bergfelt, Nogueira et al. 1999).

LH receptor expression is not detectable in granulosa cells of bovine follicles <8mm (Bao, Garverick, Smith et al. 1997). Beg et al (2001) found that there was a significant increase in LH receptor mRNA expression in the largest compared to second largest follicle 8 hours before the start of deviation when the diameter of the

largest follicle ranged from 8.0 to 8.4mm, see figure 1.4. (Beg, Bergfelt, Kot et al. 2001). Similarly, a recent study by Luo et al (2005) reported that on a day equivalent to the beginning of deviation, LH receptor mRNA expression was 8.3 times greater in the dominant follicle than in the largest subordinate follicle (Luo, Gumen, & Wiltbank 2005). In support of these findings Mihm et al (2006) used microarrays to show that during the early growth phase of first-wave dominant follicles in cattle there was a marked increase in LH receptor mRNA in granulosa cells which was concomitant with a reduction in expression of FSH mRNA and other FSH-induced genes (Mihm, Baker, Ireland et al. 2006). Despite these results there is still some discrepancy on the proposed degree of involvement of LH receptor levels in the selection of the dominant follicle as some studies reported no differences in LH receptor expression levels between the largest and second largest follicles until after selection of the dominant follicle (Bodensteiner, Wiltbank, Bergfelt et al. 1996; Evans & Fortune 1997), Regardless of this controversy, it is generally agreed that LH is required for the continued development of the dominant follicle during deviation.

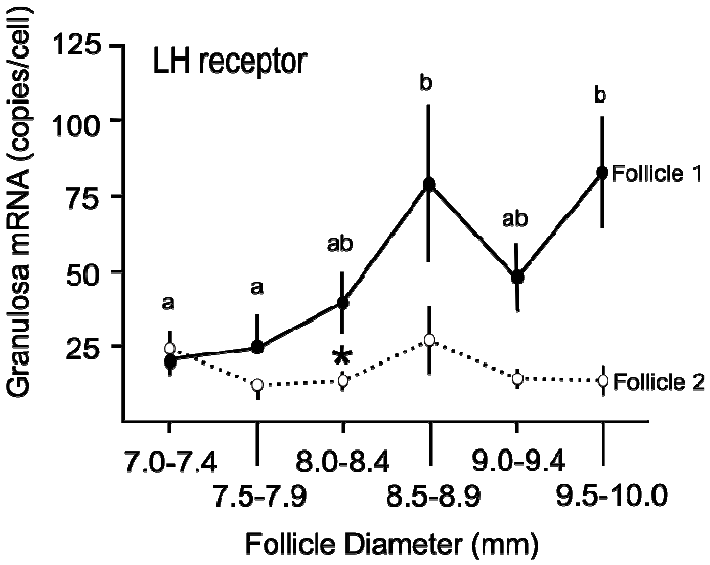


Figure 1.4. Granulosa LHR mRNA content of largest (Follicle 1) and second largest (Follicle 2) bovine follicles. Means with different letters (a, b) within a follicle are different ($P < 0.05$). An asterisk indicates the beginning of mean differences between follicles. Adapted from (Beg, Bergfelt, Kot, et al. 2001).

Other endocrine regulators. Nutritional status in cattle is believed to have a significant effect on circulating growth hormone (GH) levels and dietary restriction has been shown to increase plasma concentrations of GH (Reviewed by Armstrong 1996). Treatment of cattle with GH has been found to have a significant effect on follicle development (Gong, Bramley, & Webb 1993; Lucy 2003; Lucy, Bilby, Kirby et al. 1999). In cattle, the main effect of GH seems to be through an increase in circulating concentrations of insulin-like growth factor (IGF) (Gong, Baxter, Bramley et al. 1997) which have well-characterised effects on follicle development (see below).

Both severe under-nutrition and obesity are associated with impaired folliculogenesis (Kendall, Gutierrez, Scaramuzzi et al. 2004). Leptin, a metabolic hormone that modulates feeding behaviour (Foster & Nagatani 1999) has been demonstrated to inhibit the synergistic relationship between gonadotrophins and insulin in cattle (Spicer 2001), therefore, leptin may affect the process of follicle selection indirectly by reducing the efficiency of steroidogenesis in developing follicles (Kendall, Gutierrez, Scaramuzzi et al. 2004).

Several other substances have been reported to regulate follicle selection including prolactin (McNeilly, Glasier, Jonassen et al. 1982), insulin (Diskin, Mackey, Roche et al. 2003), and numerous neuropeptides (Diskin, Mackey, Roche et al. 2003).

1.1.3.2 Local regulatory mechanisms

Many follicular growth factors and cytokines are thought to regulate selection of the dominant follicle. The following is a description of those factors for which roles in selection have been well-described.

IGF system. The IGF system is composed of two IGF peptides (IGF-I and IGF-II), two IGF receptors (IGFR1 and IGFR2), six different and specific IGF binding proteins (IGFBP-1, -2, -3, -4,-5 and -6) and IGFBP proteases (kallikreins, cathepsins and matrix metalloproteinases (e.g.pregnancy-associated plasma protein-A, PAPP-A) (Reviewed by Spicer & Echternkamp 1995). The actions of IGFs are primarily

exerted through IGFR-1 which has a high degree of homology with the insulin receptor. The IGFR-2 has no known intrinsic signalling function but it is thought to act as a negative regulator of IGF activity by sequestration, endocytosis and degredation of IGF-II (Jones & Clemmons 1995). The IGF system is known to be involved in follicle development in mammalian (Reviewed by Mazerbourg, Bondy, Zhou et al. 2003) and non-mammalian (Armstrong & Hogg 1996) species.

IGF-I and IGF–II. IGFs act synergistically with gonadotrophins to stimulate proliferation, differentiation and steroidogenesis in follicular cells (Adashi, Resnick, Payne et al. 1997; Gutierrez, Campbell, & Webb 1997a; Hammond, Mondschein, Samaras et al. 1991; Monniaux, Monget, Besnard et al. 1997; Spicer, Alpizar, & Echternkamp 1993). IGFs induce these effects by increasing both the number of gonadotrophin receptors and the activity of gonadotrophin receptor second messenger signalling cascades (Reviewed by Adashi 1998).

The major source of circulating IGF-I is the liver. Yuan et al (1998) detected both IGF-I and -II in bovine granulosa cells from dominant follicles using in situ hybridisation, where levels of IGF expression were greater in the dominant compared to subordinate follicles (Yuan, Bao, Garverick et al. 1998). Similarly Spicer et al (1995) reported the detection of both IGF-I and –II in bovine granulosa and theca cells (Spicer & Echternkamp 1995). These results are in contrast with the findings of Armstrong et al (2000) and Perks et al (1999) who were unable to detect IGF-I in granulosa or theca cells but were able to detect IGF-II in theca but not granulosa cells (Armstrong, Gutierrez, Baxter et al. 2000; Perks, Peters, & Wathes 1999). Similarly Sudo et al (2007) found no mRNA encoding IGF-I in granulosa cells at all stages of development (Sudo, Shimizu, Kawashima et al. 2007) and it has ben reported that bovine granulosa cells did not produce IGF-I in serum-free culture (Gutierrez, Campbell, Armstrong et al. 1997a).

To date a number of studies have suggested a role for the IGF system in the selection of the dominant follicle in monovular species. Establishment of dominance is associated with higher concentrations of IGF-I but lower levels of IGFBPs in the

dominant follicle than subordinate follicles in cattle and horses (Beg, Bergfelt, Kot et al. 2002). Santiago et al (2005) found that bovine dominant follicles had an 8.1-fold higher concentration of free IGF compared to subordinate follicles (Santiago, Voge, Aad et al. 2005). Intra-ovarian administration of IGF-I for 7 days directly into bovine ovaries resulted in an increase in the size of the largest but not the second largest follicle (Spicer, Alvarez, Prado et al. 2000). Some of the most convincing evidence of the involvement of IGF-I in follicle selection has come from in vivo studies using injection of IGF-I into individual follicles in mares and heifers. Injection of recombinant human IGF-I (rhIGF-I) directly into the second largest follicle of a wave at the expected beginning of deviation in mares resulted in the continuous growth of the injected follicle which became dominant and eventually ovulated (Ginther, Gastal, Gastal et al. 2004b). In contrast, injection of the future dominant follicle with IGFBP-3 resulted in a 90% reduction in free IGF-I levels and in the injected follicle becoming subordinate rather than dominant (Ginther, Gastal, Gastal et al. 2004a). Taken together these results indicate that IGF-I is a strong candidate for involvement in the process of follicle selection.

IGFBPs and IGFBP Proteases. Six types of IGFBPs (1 to 6) and at least 51 different IGFBP isoforms have been identified in bovine follicular fluid (Nicholas, Scougall, Armstrong et al. 2002). IGFs have been observed by immunohistochemistry to co-distribute with IGFBPs and thus IGFBPS may provide a mechanism for extra-cellular storage of IGFs (Hill, Clemmons, Wilson et al. 1989). Changes in IGFBP concentrations have been recorded during follicle growth and development in cattle (de la Sota, Simmen, Diaz et al. 1996; Stewart, Spicer, Hamilton et al. 1996). These changes in binding protein levels occur through differential regulation of binding protein expression, alterations in protease activity or by selective uptake of binding proteins from circulation during follicle growth (Canty, Boland, Evans et al. 2006).

Follicle dominance is normally associated with decreased activity of low molecular weight binding proteins, IGFBP-4 and IGFBP-5 (Austin, Mihm, Evans et al. 2001) (Rivera & Fortune 2003a). PAPP-A is a 200kDa metalloprotease that has been shown to be an important regulator of IGF bioavailability through its proteolytic

activities on IGFBPs (Sudo, Shimizu, Kawashima et al. 2007). Work done in cattle by Rivera et al (2003) found that in the early dominant follicle, intrafollicular concentrations of PAPP-A and free-IGF-I increased, whilst levels of IGFBP-4 and IGFBP-5 were reduced (Rivera & Fortune 2003a). In another study by the same author it was demonstrated that PAPP-A accounted for all of the proteolytic activity against IGFBP-4 and made a significant contribution to the proteolytic activity against IGFBP-5 in follicular fluid from bovine pre-ovulatory follicles (Rivera & Fortune 2003b). There were no changes in IGFBP-4 gene expression throughout follicle development and therefore proteolysis of IGFBP-4 is considered to be the primary mechanism involved in the regulation of this binding protein (Canty, Boland, Evans et al 2006).

There are several reports of decreased levels of IGFBP-2 in follicles before or after they become dominant compared to subordinate or atretic follicles (Armstrong, Baxter, Gutierrez et al. 1998; Canty, Boland, Evans et al 2006; Nicholas, Scougall, Armstrong et al 2002; Roberts & Echternkamp 2003; Santiago, Voge, Aad et al 2005; Schams, Berisha, Kosmann et al. 2002; Yuan, Bao, Garverick et al 1998). Follicular fluid levels of IGFBP-2 may be largely regulated at the transcriptional level (Canty, Boland, Evans et al 2006), however some regulation may be attributable to the protease activity of PAPP-A and other proteases (Monget, Fabre, Mulsant et al. 2002).

Levels of IGFBP-3 tended to remain constant throughout follicle development in most species and expression was observed mainly in the thecal layer of growing follicles (Canty, Boland, Evans et al 2006; Monget, Fabre, Mulsant et al. 2005). Canty et al (2006) suggested that most of the observed IGFBP-3 expression may originate from systemic circulation (Canty, Boland, Evans et al 2006).

Oestradiol. In addition to its endocrine role of suppression of FSH levels during follicle deviation, follicular oestradiol has important paracrine roles in promoting the development of the dominant follicle. These roles are mediated through the mitogenic effects of oestradiol as well as through its effects on enhancing

gonadotrophin-induced responses in follicular cells such as expression of gonadotrophin receptors and steroidogenic enzymes (Reviewed by Britt & Findlay 2002; Rosenfeld, Wagner, Roberts et al. 2001). It has also been demonstrated in sheep and pigs that oestradiol promotes IGF-I production (Spicer & Chamberlain 2000). Oestradiol levels have been shown to increase differentially in the largest follicle compared to the second largest follicle at the beginning of diameter deviation in cattle and horses (Beg, Bergfelt, Kot et al. 2001a; Ginther, Beg, Kot et al. 2003; Ginther, Kot, Kulick et al. 1997b; Mihm, Austin, Good et al. 2000) and this increase is driven, at least in part, by the increase in IGF-I bioavailability in the largest follicle (Ginther, Bergfelt, Beg et al. 2004).

Androgens and Progestins. There were no significant differences in follicular fluid concentrations of androgens among the three largest follicles in cattle prior to diameter deviation (Beg, Bergfelt, Kot et al 2002). Furthermore, concentrations of androgens did not change in the second largest follicle following ablation of the largest follicle just before the onset of deviation (Beg, Bergfelt, Kot et al 2002). Similar results were observed in the horse (Donadeu & Ginther 2002). These results led to the conclusion that androgens are not involved in the beginning of deviation. After the beginning of deviation, levels of androgens were observed to increase in dominant follicles and decrease in subordinate follicles of cattle (Beg, Bergfelt, Kot et al 2001b; Beg, Bergfelt, Kot, et al. 2002; Stewart, Spicer, Hamilton et al 1996). This was in contrast with the horse where androgens increased in subordinate but not dominant follicles after deviation (Donadeu & Ginther 2002). Although androgens are required as a substrate for FSH-dependent oestradiol synthesis and so function indirectly to stimulate follicle growth, they have also been shown to directly and indirectly promote follicle atresia (Reviewed by Walters, Allan, & Handelsman 2008). Results regarding the involvement of progesterone in follicle selection are inconsistent. Some studies reported no changes in progesterone levels at the beginning of deviation (Evans & Fortune 1997; Mihm, Good, Ireland et al 1997), whereas others did observe an increase in progesterone in the largest compared to the second largest follicle at the expected beginning of deviation (Beg, Bergfelt, Kot et al 2001a; Ginther, Bergfelt, Beg et al. 2001b). Whatever its role during the early

development of the dominant follicle, progesterone is recognised to play a critical role in ovulation. Mice that are deficient in progesterone receptor (PR) fail to ovulate (Robker, Russell, Espey et al. 2000). Until recently the function of progesterone in the ovulatory process was largely unknown. It has recently become clear that the LH surge together with the action of progesterone and its cognate receptor induces a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-1 gene expression (Robker, Russell, Espey et al. 2000) which is thought to cleave versican (anti-adhesive proteoglycan) in the expanded cumulus-oocyte-complex (COC) thus allowing the COC to be released (Espey & Richards 2006).

Inhibin, Activin, Follistatin. Inhibins and activins are dimeric glycoproteins that are members of the TGF-β superfamily. Different inhibin and activin forms result from combinations of α- and β- (βA and βB) subunits. There are two known forms of inhibin, each composed of different β subunits: Inhibin A (α-βA) and Inhibin B (α-βB) (Reviewed by Knight & Glister 2001; Phillips 2005). β subunit dimerisation results in 3 possible forms of activin: Activin A (βA-βA), Activin AB (βA-βB) and Activin B (βB-βB). Follistatin is a cysteine-rich monomeric glycoprotein that is structurally unrelated to activins and inhibins and that binds with high affinity to inhibin/activin β subunits. Follistatin binds to inhibins with much less affinity than to activins and seemingly without affecting inhibin activity.

In addition to the systemic FSH-suppressing effects of inhibin, a number of in vitro studies have demonstrated local-paracrine roles for granulosa cell-derived activin, inhibin and follistatin in follicle development throughout effects on gonadotrophin responsiveness, steroidogenesis, oocyte maturation, ovulation and CL function (Reviewed by Knight & Glister 2001).

Activin and inhibin are produced in large amounts by the growing dominant follicle, although this increased production only occurs after the beginning of deviation (Beg, Bergfelt, Kot et al 2002). One study demonstrated that following ablation of the largest follicle at the start of deviation, the concentration of activin A in the second largest follicle increased (Ginther, Beg, Bergfelt et al. 2002). In another

study, an increase in the activin/inhibin ratio was identified in bovine follicles as they increased in diameter from 3 to 6mm (Glister, Groome, & Knight 2006). During this increase in follicle size there was an increase in activin A by approximately 30-fold compared to only a 6-fold increase in inhibin A. The concentration of follistatin remained constant throughout this time. Given that follistatin binds activin A in a ratio of 2:1, follistatin concentration would exceed that of activin A until after follicles grew larger that 6mm (Glister, Groome, & Knight 2006), just before the beginning of follicle deviation. Taken together these results suggest an important role for activin in the beginning of deviation**.**

1.1.4 Control of ovulation and follicle-to-luteal transition

The ovulatory process is complex involving reactivation of oocyte meiosis, rupture of the apical follicle wall and, finally, oocyte release. Subsequent tissue remodelling and cell differentiation processes result in the formation of the corpus luteum (CL). For over a century it was believed that the process of ovulation was induced by increased follicular pressure, thought to be caused by the action of smooth muscle in the ovary (Asdell 1962). In 1963, however, it was demonstrated that intrafollicular pressure did not increase prior to ovulation (Blandau & Rumery 1963; Espey & Lipner 1963). In addition the ovary was not found to have any functional muscle tissue (Espey 1978). The general consensus is now that ovulation is an acute inflammatory reaction that is induced by the ovulatory gonadotrophin surge (Reviewed by Espey & Richards 2006). A great deal of focus over the past few decades has been placed on the critical role of prostaglandins (classically known activators of inflammatory response) in the ovulatory process (Reviewed by (Murdoch, Hansen, & McPherson 1993; Olofsson & Leung 1996). Postaglandins are synthesised from arachidonic acid which is formed from cell membrane phospholipids. The rate limiting step in prostaglandin synthesis is believed to be controlled by the COX enzymes, of which there are two main isoforms. COX1 is a constitutive enzyme that is present in almost all mammalian tissues whereas COX2, described as an inducible form, is present at low/undetectable levels in most tissue types. As a consequence of the ovulatory LH surge, prostaglandin synthesis is

induced and COX2, which is expressed by granulosa and cumulus cells, is upregulated (Murdoch, Hansen, & McPherson 1993). A possible correlation between a peak in COX2 expression and duration of ovulation has been proposed (Duffy & Stouffer 2001; Richards 1997). Studies in the rat reported that the induction of COX2 following hCG was rapid (within 2-4 hours) and this occurred 10 hours prior to ovulation (Sirois & Richards 1992). In the cow, the induction of COX2 was not as rapid and occurred approximately 18 hours after hCG treatment and approximately 10 hours before follicle rupture (Sirois 1994). In the horse, COX2 induction was found to occur 30 hours after hCG treatment and once again the interval of time between COX2 induction and follicle rupture was 10 hours (Sirois & Dore 1997). These results supported the hypothesis that "COX2 may be a molecular determinant that sets the alarm of the mammalian ovulatory clock" (Richards 1997).

Recently, a great deal of effort has been made to identify the genes that in addition to COX2 are involved in ovulation. Candidate genes have been identified in mural and cumulus granulosa cells, theca cells and the oocyte itself. A comprehensive description of genes involved in ovulation can be found elsewhere (Espey & Richards 2006).

1.2 Vascular Endothelial Growth Factor (VEGF) and its role in folliculogenesis

1.2.1 Discovery

VEGF, initially named Vascular Permeability Factor (VPF), was first identified in the early 1980's as a secretory product of tumour cells that was observed to induce vascular leakage (Senger, Galli, Dvorak et al. 1983) leading to the hypothesis that VPF could mediate the permeability of blood vessels in tumour development. For a number of years after its discovery, VPF/VEGF remained undefined at the molecular level. Ferrara and associates (1989) reported the isolation of VEGF, a diffusible endothelial cell-specific mitogen from bovine pituitary follicular cell-conditioned medium (Ferrara & Henzel 1989). They demonstrated that VEGF was able to induce

proliferation of endothelial cells in addition to promoting endothelial cell migration and survival. Connolly and associates sequenced VPF and subsequently discovered that VPF and VEGF were in fact identical molecules (Connolly, Olander, Heuvelman et al. 1989).

1.2.2 VEGF Family

VEGF is a member of the cysteine knot family of growth factors and is secreted as a dimer in which two VEGF monomers are linked together by two disulphide bridges (Potgens, Lubsen, van Altena et al. 1994). The VEGF family is structurally related to the Platelet-Derived Growth Factor (PDGF) family and includes several members including VEGF-A (usually referred to as VEGF), VEGF-B, VEGF-C, VEGF-D and Placental Growth Factor (PlGF) (reviewed in Tammela, Enholm, Alitalo et al. 2005). The role of VEGF-B is not yet fully understood, although high levels of expression have been observed in skeletal muscle, myocardium and brown fat which may link the actions of VEGF-B with high energy metabolism. VEGF-C and –D have both been implicated in the growth of lymphatic vessels (lymphanogenisis). The main sites of expression of PlGF include the placenta, heart and lungs (Persico, Vincenti, & DiPalma 1999). Potential roles proposed for PlGF include the mediation of arteriogenesis (increased diameter of existing blood vessels) and formation of arteries from pre-existing arterioles (Tammela, Enholm, Alitalo et al. 2005). Other highly related proteins, VEGF-E and VEGF-F, have also been identified. VEGF-E is encoded by pox viruses of the Orf and psuedocowpox families (Meyer, Clauss, Lepple-Wienhues et al. 1999) and VEGF-F variants have been isolated from the venom of snakes (Takahashi, Hattori, Iwamatsu et al. 2004).

The gene encoding VEGF-A (VEGF) is located on the short arm of chromosome 6 in the human (Zimmermann, Xiao, Bohlen et al. 2002), on chromosome 17 in the mouse (Gomez, Simon, Remohi et al. 2002) and chromosome 23 in the cow (http://www.ncbi.nlm.nih.gov). The human VEGF-A (VEGF) gene is made up of eight exons, separated by seven introns and alternative splicing of VEGF results in multiple isoforms (VEGF-$_{121,\ 145,\ 148,\ 162,\ 165,\ 165b,\ 183,\ 189\ \text{and}\ 206}$) that have unique

signalling and functional properties (see figure 1.5) (Yamazaki & Morita 2006). Each of these isoforms are named according to the number of amino acids in the mature protein sequence. In both murine and bovine species VEGF isoforms contain one less amino acid than in humans, thus the murine and bovine homologues of $VEGF_{165}$ are $VEGF_{164.}$ All VEGF isoforms identified so far contain exons 1 to 5 and different combinations of exons 6 to 8. Exons 6 and 7 encode two heparin binding sites which affect receptor binding and isoform solubility. Isoforms containing exon 6 (e.g. $VEGF_{145,}$ $VEGF_{189,}$ $VEGF_{206}$) are tightly bound to the cell membrane, whereas isoforms that lack exon 6 (e.g. $VEGF_{165}$) are diffusible (not membrane-bound). Isoforms that lack both exons 6 and 7 (e.g. $VEGF_{121}$) are even more diffusible than those that only lack exon 6. $VEGF_{165}$ is the physiologically most common isoform of VEGF (Cross, Dixelius, Matsumoto et al. 2003).

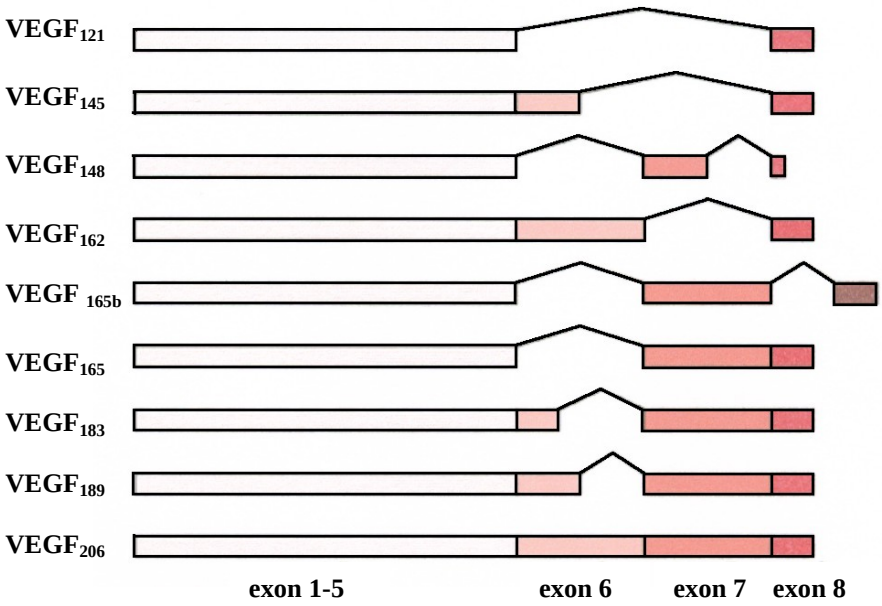


Figure 1.5. Different isoforms resulting from alternative splicing of the VEGF gene. Adapted from (Takahashi & Shibuya 2005).

1.2.3 VEGF Receptors

VEGF can bind to a variety of receptors on the cell surface (see figure 1.6) including the receptor tyrosine kinases (RTKs), VEGFR1, VEGFR2 and VEGFR3, and neuropilins-1 and -2 (NRP1, NRP2) (Reviewed in Cebe-Suarez, Zehnder-

Fjallman, & Ballmer-Hofer 2006; Petrova, Makinen, & Alitalo 1999; Takahashi & Shibuya 2005) . RTKs are characterised by seven immunoglobulin (Ig) homology domains in the extracellular ligand-binding region, a single transmembrane domain, and an intracellular tyrosine kinase domain that is interrupted by a 65 to 97-long stretch of hydrophilic residues that make up a kinase-insert domain (Reviewed by van der, Hunter, & Lindberg 1994). This domain is important for substrate recognition. Neuropilin-1 (NRP-1) and Neuropilin-2 (NRP-2) were first described as receptors for type 3 Semaphorins, which have important roles in the control of axonal growth and angiogenesis (Neufeld, Cohen, Shraga et al. 2002). Subsequently it was found that the neuropilins also function as co-receptors for some forms of VEGF. NRP-1 binds to VEGF-A, VEGF-B and PIGF, whereas NRP-2 binds to VEGFA, VEGF-C and PIGF (Reviewed by Otrock, Makarem, & Shamseddine 2007).

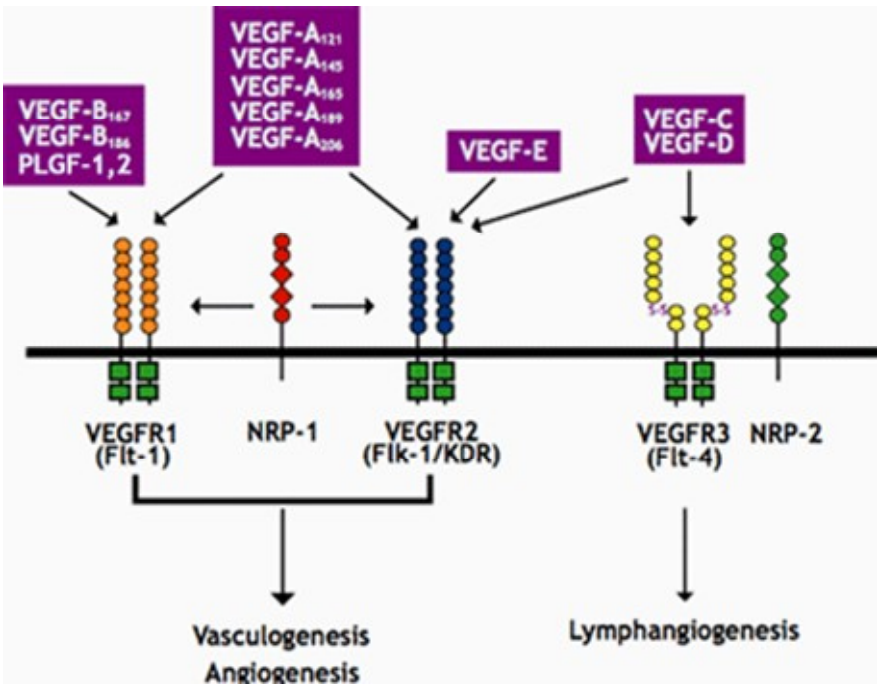


Figure 1.6. Members of the VEGF family and their putative cognate cell membrane receptors.Taken from (Hicklin & Ellis 2005).

1.2.3.1 VEGFR1

VEGFR1 (fms-like tyrosine kinase, Flt-1) was the first RTK to be identified as a VEGF receptor almost 20 years ago (Shibuya, Yamaguchi, Yamane et al. 1990). This 180 kDa glycoprotein has a high affinity for VEGF, VEGF-B, PlGF, and TfsvVEGF (Trimeresurus flavoviridis snake venom VEGF). Immunoglobulin domain 2 of VEGFR1 is the major binding site for VEGF and PlGF (Takahashi & Shibuya 2005). VEGFR1 binds VEGF with at least 10-fold higher affinity than VEGFR2 (Terman, Dougher-Vermazen, Carrion et al. 1992). However, the tyrosine kinase activity of this receptor is relatively weak compared to VEGFR2. The biological function of VEGFR1 remains unclear. It has been proposed that VEGFR1 acts as a decoy receptor rather than as a signal transducer (Park, Chen, Winer et al. 1994). VEGFR1 has been demonstrated to negatively regulate angiogenesis during early development, with targeted deletion of the VEGFR1 gene resulting in early embryonic death in the mouse (between days 8.5 and 9.5) due to overgrowth of endothelial cells (Fong, Rossant, Gertsenstein et al. 1995). Mice that express the extracellular region of VEGFR1 but lack the kinase domain develop normal vasculature and survive (Hiratsuka, Minowa, Kuno et al. 1998), thus indicating that the tyrosine kinase domain of VEGFR1 is not required for embryonic development. In contrast to the embryo, VEGFR1 has been found to enhance angiogenesis in a number of pathological conditions during postnatal life (Takahashi & Shibuya 2005), thus demonstrating that the actions of VEGFR1 can differ depending on the stage of development and cell type in which it is expressed.

1.2.3.2 VEGFR2

VEGFR2 (also known as KDR or Flk-1) is a 200 kDa glycoprotein that binds VEGF, VEGF-C and VEGF-D. Although VEGF has a lower affinity to VEGFR2 than to VEGFR1, it has been shown that VEGFR2 is the primary receptor through which VEGF signalling occurs in endothelial cells (Gille, Kowalski, Li et al. 2001). In addition to endothelial cell types, VEGFR2 is expressed in numerous non-

endothelial cell types such as neurons, osteoblasts, pancreatic cells, hematopoetic stem cells and ovarian cells (Tammela, Enholm, Alitalo et al. 2005). The key role of this receptor in development is demonstrated by a failure of Flk-1-null mice to develop blood islands and organised blood vessels. Such gene deletion causes embryonic death between days 8.5 and 9.5 (Shalaby, Rossant, Yamaguchi et al. 1995) thus showing that VEGFR2 is an absolute requirement for vascular development. VEGFR2 is considered to be the major mediator of both physiological and pathological effects of VEGF. These include cell proliferation, survival, migration and permeability. Proliferation of endothelial cells is stimulated through the classical RTK-activated Extracellular Regulated Kinase (ERK) pathway as well as activation of PLCγ-1 (Takahashi, Yamaguchi, Chida et al. 2001). VEGFR2 has been shown to promote survival of endothelial cells via the activation of PI-3 kinase/Akt pathway (Reviewed by Cebe-Suarez, Zehnder-Fjallman, & Ballmer-Hofer 2006).

1.2.3.3 VEGFR3

VEGFR3 (also known as fms-like tyrosine kinase, Flt-4) has 6 rather than 7 Ig domains, as the fifth Ig domain is cleaved post-translationally. VEGFR3 binds VEGF-C and VEGF-D. This receptor is expressed on all endothelial cell types during development but becomes confined to lymphatic endothelial cells in the adult (Kaipainen, Korhonen, Mustonen et al. 1995). However, VEGFR3 is upregulated in the microvasculature of tumours and wounds. Recently, (Tammela, Zarkada, Wallgard et al. 2008) have shown that blocking VEGFR3 resulted in decreased vessel sprouting and endothelial cell proliferation.

1.2.3.4 Neuropilins

NRP-1 (mw, 130-140kDa) and NRP-2 (mw, 130 kDa) are transmembrane glycoproteins with short intracellular domains that can only signal in association with other receptor types. NRP-1 can associate with both VEGFR1 (Fuh, Garcia, & de Vos 2000) and VEGFR2 (Whitaker, Limberg, & Rosenbaum 2001). The formation

of a complex between NRP-1 and VEGFR2 enhances proliferation and migration induced by $VEGF_{165}$ (Whitaker, Limberg, & Rosenbaum 2001). Targeted inactivation of NRP-1 in mice results in severe defects such as impairment of neural vascularisation and disorganised/insufficient development of vascular networks in the yolk sac (Kawasaki, Kitsukawa, Bekku et al. 1999). Following inactivation of NRP-2, there is either absence or severe reduction of small lymphatic vessels. However, arteries, veins and larger lymphatic vessels develop normally, thus suggesting that NRP-2 is selectively required for the formation of small lymphatic vessels and capillaries (Yuan, Moyon, Pardanaud et al. 2002).

1.2.4 Roles of VEGF in Angiogenesis

The biological importance of VEGF has been shown through gene deletion studies, where the loss of even a single VEGF allele was demonstrated to be lethal between embryonic days 11 and 12 (Carmeliet, Ferreira, Breier et al. 1996; Ferrara, Carver-Moore, Chen et al. 1996). Mutant embryos exhibited significant defects of the vasculature in many organs including the brain, heart, and spinal cord. These embryos also presented yolk sacs that contained a significantly reduced number of nucleated red blood cells within the blood islands. $VEGF^{-/-}$ embryos were observed to have a more severe phenotype than $VEGF^{+/-}$ (Carmeliet, Ferreira, Breier et al. 1996). Lethal mutations were also observed when VEGF was over-expressed (Feucht, Christ, & Wilting 1997; Flamme, von, Drexler et al. 1995). When VEGF was expressed in chicken embryos at a concentration 2-3 times greater than found physiologically there were lethal deleterious effects, a main effect being the prevention of normal development of the embryonic heart (Miquerol, Langille, & Nagy 2000).

The best defined role of VEGF is promotion of blood vessel formation both during normal and pathological states. Angiogenesis, the growth of blood vessels from pre-existing vessels is dependant on the balance between stimulatory pro-angiogenic and inhibitory anti-angiogenic factors. The pro-angiogenic effects of VEGF result from stimulation of proliferation, survival and migration of endothelial cells (Leung,

Cachianes, Kuang et al. 1989; Senger, Ledbetter, Claffey et al. 1996; Spyridopoulos, Brogi, Kearney et al. 1997; Takahashi & Shibuya 2005). Increased angiogenesis is a hallmark of cancer and it has been well documented that VEGF stimulates the progression of various types of cancer through its pro-angiogenic properties (Reviewed in Harmey 2004).

1.2.5 Non-Angiogenic roles of VEGF

In addition to its classical role as an endothelial cell-specific mitogen (Ferrara, Houck, Jakeman et al. 1992), VEGF (and its receptors) has been shown to have effects on a variety of non-endothelial cell types (Reviewed in Duffy, Bouchier-Hayes, & Harmey 2004; Haigh, Ruiz de Almodovar, Schneider et al. 2007). These effects involve cell proliferation, survival, and migration.

1.2.5.1 Proliferation and Survival

Cell proliferation is a key process during neural development and is also important for the regeneration of tissue following neural injury. VEGF has been shown to stimulate proliferation of a number of neural cell types including astrocytes (Silverman, Krum, Mani et al. 1999), schwann cells (Sondell, Lundborg, & Kanje 1999a; Sondell, Lundborg, & Kanje 1999b), microglia (Forstreuter, Lucius, & Mentlein 2002), and cortical neurons (Jin, Zhu, Sun et al. 2002; Zhu, Jin, Mao et al. 2003).

Another physiological process that requires intense proliferative activity is wound healing. In skin tissue, keratinocytes are considered to be the main site of VEGF production during cutaneous wound healing. Wilgus et al (2005) demonstrated that murine epidermal keratinocytes expressed VEGF and VEGFR1, and that VEGF was capable of stimulating proliferation in normal human epidermal keratinocytes (NHEKs) (Wilgus, Matthies, Radek et al. 2005). These proliferative effects were blocked following administration of anti-VEGFR1 antibodies. In addition, application of VEGFR1 antibodies directly to skin wounds resulted in reduced

number of keratinocyes at the wound edge and subsequently delayed re-epithilisation. In support of these findings, the generation of a strain of mice in which keratinocytes did not express VEGF resulted in mice that had normal skin capillary systems however, when full thickness skin wounds were made, healing was delayed compared to control mice whose keratinocytes did express VEGF (Rossiter, Barresi, Pammer et al. 2004) thus indicating that VEGF is an important component of wound healing.

Myofibroblasts are important for the growth and repair of normal tissue and it is common to find myofibroblasts at sites of infarction (macroscopic area of necrotic tissue caused by loss of adequate blood supply). Myofibroblasts isolated from infarctions in rats have been shown to express VEGF, VEGFR1 and VEGFR2, suggesting an autocrine role for VEGF, whereby VEGF contributes to the repair of damaged tissue (Chintalgattu, Nair, & Katwa 2003). In addition VEGF has been shown to promote cellular proliferation and survival in heart tissues (reviewed in Zachary, Mathur, Yla-Herttuala et al. 2000).

Systemic administration of VEGF in mice resulted in VEGFR1 receptor activation and subsequent release of hepatocyte growth factor (HGF) which induced hepatocyte proliferation and increased liver sizes (LeCouter, Moritz, Li et al. 2003). In studies by Brockhorn et al (2007) VEGF was shown to stimulate proliferation of hepatocytes and subsequently regeneration of liver tissue following a partial (70%) hepatectomy (Bockhorn, Goralski, Prokofiev et al. 2007). VEGF was unable however to stimulate liver regeneration following a 90% hepatectomy (Bockhorn, Schollmann, Opitz et al. 2007) thus indicating that although VEGF is able to stimulate hepatocyte proliferation following liver damage, the extent to which VEGF can assist with tissue regeneration depends on the amount of damage present.

VEGF is also expressed in human dental follicle cells (HDFC). VEGF was observed to stimulate HDFC proliferation and differentiation and to enhance HDFC survival (Chen, Qian, Wu et al. 2007). These results are in support of a role for VEGF in tooth eruption.

The ability of VEGF to stimulate cell proliferation has also been described in relation to cancer. The first non-endothelial tumour cell type shown to express VEGFR2 was ovarian carcinoma cells (Boocock, Charnock-Jones, Sharkey et al. 1995). Since then a wide variety of neoplasic cell types have been shown to express VEGF and its receptors (Herold-Mende, Steiner, Andl et al. 1999; Masood, Cai, Zheng et al. 2001; Stewart, Turley, Cook et al. 2003; Stitt, Simpson, Boocock et al. 1998). For example, pancreatic tumour cells express elevated levels of VEGF and VEGF receptors compared to normal pancreatic cells (Itakura, Ishiwata, Shen et al. 2000). Treatment of pancreatic cell lines (Dan-G and AsPc-1) with VEGF resulted in significant increase in cell proliferation (Von, Cramer, Hocker et al. 2000). These proliferative effects were abolished when cell lines were transfected with a retrovirus encoding a mutant non-functional form of VEGFR2 (Von, Cramer, Hocker et al. 2000). These findings indicate that VEGF stimulates proliferation of human pancreatic cells through interaction with VEGFR2. Human malignant mesothelioma is a neoplasm that develops from the lung surface, peritoneum and testes (Strizzi, Catalano, Vianale et al. 2001). Mesothelioma cells have been shown to co-express VEGFR1 and VEGFR2. Exposure of these cells resulted in a dose-dependant increase in phosphorylation of both receptors in addition to increased cell proliferation, an effect that could be inhibited by administration of VEGFR-neutralising antibodies (Strizzi, Catalano, Vianale et al. 2001).

In 2001, Oosthuyse et al reported that the presence of a mutation in the promoter region of the VEGF gene in transgenic mice resulted in the onset of neuron degeneration (Oosthuyse, Moons, Storkebaum et al. 2001). Since then, VEGF has been regarded as a trophic factor of cells of the central nervous system. VEGF is known to act directly on neural and glial cells to promote cell survival (Matsuzaki, Tamatani, Yamaguchi et al. 2001; Wick, Wick, Waltenberger et al. 2002; Jin, Mao, & Greenberg 2000a; Jin, Mao, & Greenberg 2000b; Sondell, Lundborg, & Kanje 1999b).

The largest avascular tissue in the body, the intervertebral disc (located between vertebral bodies) is composed of two tissue types, the nucleus pulposus (NP) and the annulus fibrosus (AF) (Fujita, Imai, Suzuki et al. 2008). Despite the lack of vasculature, the NP was shown to have high levels of expression of VEGF and VEGFR1. Fujita (2008) studied the role of VEGF in this tissue type and found that treatment of NP cells with a VEGF antagonist induced apoptosis, thus indicating that VEGF/VEGFR1 cascade has a survival function in NP cells (Fujita, Imai, Suzuki et al. 2008).

In addition to having an autocrine effect on proliferation of tumour cells, VEGF has also been shown to enhance tumour cell survival. Breast cancer is one example of a malignancy in which non-angiogenic VEGF signalling has been well documented (Bachelder, Crago, Chung et al. 2001; Chung, Bachelder, Lipscomb et al. 2002; Mercurio, Lipscomb, & Bachelder 2005; Pidgeon, Barr, Harmey et al. 2001).

1.2.5.2 Migration and motility

Similar to endothelial cells, VEGF can induce migration of non-endothelial cells. For example cancer cells acquire the ability to migrate and invade tissues as a mechanism of cancer progression. VEGF is able to induce migration of these cells via autocrine mechanisms (Mercurio, Lipscomb, & Bachelder 2005). NRP-1 was essential for invasion of breast cancer cells via CXCR4 (a chemokine receptor), which is regulated by VEGF (Bachelder, Crago, Chung et al. 2001). Another study by Bachelder and co-workers (2002) assessed the effects of VEGF inhibition (using antisense oligonucleotides) on the invasion and migration of cells from the MDA-MB-231 breast cancer cell line through matrigel (Bachelder, Wendt, & Mercurio 2002). Results showed that inhibition of VEGF resulted in a significant (65%) reduction in matrigel invasion. This effect was shown to be specifically caused by VEGF because re-administration of VEGF restored the migratory abilities of the breast cancer cells (Bachelder, Wendt, & Mercurio 2002).

Further evidence that VEGF is involved in migratory mechanisms comes from studies with bone tissue. Bone repair is a multi-step process that involves migration in addition to proliferation, differentiation and activation of a variety of cell types (Street, Bao, deGuzman et al. 2002). VEGF and its receptors are important regulators of angiogenesis during bone formation (ossification). In addition to this angiogenic effects, there is also evidence that VEGF may have direct effects on bone-forming osteoblasts. Osteoblasts have been found to express both VEGFR1 and VEGFR2 (Deckers, Karperien, van der et al. 2000) and to synthesise VEGF in culture (Wang, Yamazaki, Nohtomi et al. 1996). VEGF has been reported to bind to osteoblasts in culture and induce osteoblast migration and alkaline phosphatase activity (a biochemical indicator of bone turnover) (Midy & Plouet 1994). In contrast to bone formation by osteoblasts, bone resorption is carried out by osteoclasts. Bone resorption occurs in inflamed joints and is a main cause of arthritic joint disease. VEGF has been shown to act as a chemoattractant for osteoclasts (Engsig, Chen, Vu et al. 2000; Zelzer, McLean, Ng et al. 2002).

In the bovine oviduct, VEGF has been proposed to be involved in embryo transport. This was demonstrated in cultured bovine oviductal epithelial cells (BOEC) that were treated with VEGF (Wijayagunawardane, Kodithuwakku, Yamamoto et al. 2005). VEGF, alone or in synergy with LH, stimulated the production and release of substances regulating oviductal contractility, including Prostaglandin E2 (PGE2), Prostaglandin F2α (PGF2α) and Endothelin-1 (ET-1). Their results indicated that the ovulatory LH surge upregulates oviductal VEGF, which subsequently induces the releases the aforementioned substances stimulating the transport of the embryo to the uterus.

1.2.6 Roles of VEGF in Follicle Development

1.2.6.1 VEGF expression during follicle development

In most species, there is a lack of VEGF expression in primordial follicles. Typically, VEGF only becomes detectable in granulosa and theca cells when follicle development reaches the secondary stage, concurrent with the development of follicle vasculature (Fraser 2006). In primates, there is generally a further increase in VEGF mRNA in granulosa cells of late secondary and tertiary follicles, resulting in higher VEGF levels than in the theca layer in those follicles (Fraser 2006). Close to the time of ovulation, VEGF expression in granulosa cells of the primate disappeared whereas VEGF expression in the theca increased (Wulff, Wiegand, Saunders et al. 2001). This observation is in agreement with studies in the pig, where there was an increase in follicular fluid VEGF concentration during follicle growth followed by a decrease after hCG administration until VEGF became undetectable by 36 hours post hCG (Barboni, Turriani, Galeati et al. 2000). Similar patterns of expression were observed in the bovine, where VEGF expression increased with the growth of antral follicles (Greenaway, Connor, Pederson et al. 2004).

1.2.6.2 Effects of VEGF on preantral follicles

VEGF has been proposed to stimulate the growth of preantral follicles in the rat ovary (Danforth, Arbogast, Ghosh et al. 2003). In that study, direct intraovarian injection of VEGF resulted (within 24 hours) in an approximately 75% increase in primary and secondary follicles in VEGF- compared to saline-treated rats. Several explanations for the VEGF-induced increase in number of preantral follicles were proposed; VEGF might stimulate vascular supply to these follicles which would subsequently increase the delivery of nutrients and growth factors to these follicles. Alternatively, VEGF may directly stimulate proliferation/survival pathways in follicular cells resulting in a large proportion of follicles being rescued from atresia. Similar results were reported by Yang and Fortune (2007) who found that treatment of cultured pieces of bovine foetal ovarian cortex with VEGF promoted the transition

from primary to secondary follicles (Yang & Fortune 2007). Regardless of whether the observed responses of preantral follicles to VEGF in these studies involved effects on endothelial cells, non-endothelial cells or both, these results indicate that VEGF is involved in early follicle growth

1.2.6.3 Effects of VEGF on follicle selection and ovulation

Zimmerman et al (2001) showed that systemic treatment of rhesus monkeys with VEGF-neutralising antibodies during the late follicular phase resulted in delayed ovulation (Zimmermann, Xiao, Husami et al. 2001). The same authors carried out further investigations by administering VEGFR2 antibody early in the follicular phase. This resulted in arrested development in the cohort of growing follicles and subsequent lengthening of the follicular phase (Zimmermann, Xiao, Bohlen et al. 2002). This group then went on to examine whether VEGF/VERFR-2 pathway was required for gonadotrophin-dependent follicle development. This was tested in hypophysectomised mice. (Zimmermann, Hartman, Kavic et al. 2003). In the presence of VEGFR2-neutralising antibodies, gonadotrophins were unable to stimulate follicle growth to the pre-ovulatory stage. It was therefore concluded that the interaction between VEGF and VEGFR2 is required for gonadotrophins to effectively stimulate follicle development (Zimmermann, Hartman, Kavic et al. 2003). In another study, Hazzard et al (2002) demonstrated that VEGF is required for successful ovulation. This group carried out direct injections of soluble VEGFR1 peptide into preovulatory follicles and this suppressed ovulation and luteinisation leading to reduced corpus luteum function (Hazzard, Xu, & Stouffer 2002). The role of VEGF throughout follicle development has also been evaluated by administration of VEGF Trap R1R2. This is a compound comprised of the extracellular VEGFR1 and VEGFR2 domains fused with human Fc, the proteolytic fragment of an an antibody molecule that contains the two C-terminal domains of its two heavy chains. This trap is able to block responses to VEGF A, B and P1GF but not to VEGF C and D (Fraser 2006). When administered by subcutaneous injection in marmosets throughout the follicular phase, VEGF Trap R1R2 resulted in a ~80% decrease in endothelial cell area in secondary and tertiary follicles. Following inhibition of

VEGF, tertiary follicles developed but did not reach preovulatory size (>2mm) (Wulff, Wilson, Wiegand et al. 2002). The conclusions drawn from this study were that follicle development is dependant on the presence of normal thecal vasculature (Wulff, Wilson, Wiegand et al. 2002). VEGF trap was also found to affect corpus luteum function, whereby injection of the trap in the late follicular phase inhibited the rise in progesterone that is required for luteal function (Fraser, Wilson, Morris et al. 2005).

Given that VEGF inhibition prevents normal follicle growth, other studies have tested whether administration of VEGF may enhance follicle development. Shimizu and associates (2003) performed in vivo injection of VEGF gene fragments directly into the medulla of ovaries of pigs (Shimizu, Jiang, Iijima et al. 2003). VEGF was shown to be incorporated into granulosa cells following administration and concentrations of VEGF in follicular fluid in VEGF-treated ovaries were elevated in comparison with ovaries treated with saline. VEGF treatment resulted in a 2-fold increase in vascular density compared to control ovaries. This increase in vasculature was associated with a decrease in the number of atretic follicles in VEGF-treated ovaries. In addition, following injection of VEGF gene fragments, VEGFR1 mRNA was found to increase to a greater extent in theca cells than did VEGFR2, suggesting that perhaps VEGFR1 has a predominant role in thecal vasculature development (Shimizu, Jiang, Iijima et al 2003). More recently, a similar experiment in rats was carried out where VEGF gene fragments were injected into the ovaries of immature rats. Similar to observations in pigs, VEGF gene fragments resulted in increased development of thecal vasculature which subsequently led to stimulation of antral follicle development (Shimizu, Iijima, Miyabayashi et al. 2007). These results are in agreement with another study in rats, where intraperitoneal injection of VEGF in cycling rats stimulated follicle development and resulted in more oocytes being ovulated compared to untreated controls (Iijima, Jiang, Shimizu et al. 2005). Co-treatment with an inhibitor of angiogenesis (TNP-470) completely inhibited the effects of VEGF on ovulation (Iijima, Jiang, Shimizu et al. 2005). In another study, co-administration of VEGF gene fragments with GDF-9, a growth factor secreted by

the oocyte, did not augment the effects of VEGF administered alone (Shimizu, Iijima, Ogawa et al. 2008).

Studies in cattle and horses have considered the effects of VEGF specifically at the beginning of deviation during a follicular wave. In the horse, blood flow in the largest follicle was observed to increase relative to the second largest follicle approximately 1 to 2 days before the beginning of diameter deviation between the two follicles (Acosta, Gastal, Gastal et al. 2004; Gastal, Donadeu, Gastal et al. 1999). A study using coloured Doppler ultrasonography in cattle showed that detectable blood flow was not different between the largest and second largest follicles before the beginning of deviation (Acosta, Hayashi, Matsui et al. 2005). In contrast, after the beginning of deviation, blood flow was significantly increased in the dominant follicle (Acosta, Hayashi, Matsui, & Miyamoto 2005). These findings are in agreement with a study done in sheep which found that blood flow velocity was greater in the dominant follicle than in non-dominant follicles (Brown & Driancourt 1989) These results suggest that blood may be delivered preferentially to the dominant follicle. As indicated above, follicular-fluid levels of VEGF increase during growth of antral follicles and are highest in dominant-size follicles (Greenaway, Connor, Pedersen et al. 2004). In horses, the concentration of VEGF in the largest follicle was found to be higher than in the second largest follicle 24 hours after the beginning of deviation (Ginther, Gastal, Gastal, & Beg 2004a). Another study by the same authors found that injection of rh-IGF-I into the second largest follicle in the mare induced an increase in the concentration of VEGF within 24 hours (Ginther, Gastal, Gastal et al. 2004b).

Taken together, the results provide strong evidence that a sufficient vascular supply is required for successful selection and maturation of dominant follicles and that VEGF treatment can significantly enhance the development of the thecal vascular supply and protect against follicular atresia.

1.2.6.4 Effects of VEGF on granulosa cell function

Considering the demonstrated non-angiogenic roles of VEGF in a variety of tissues, it is not known to what extent the responses of follicles to manipulation of VEGF levels are due to effects of VEGF on follicular cells other than endothelial cells. In that regard, granulosa cells represent the major source of follicular VEGF in a number of species (Grasselli, Basini, Bussolati et al. 2002; Greenaway, Connor, Pedersen et al. 2004; Kamat, Brown, Manseau et al. 1995). The fact that these cells express both VEGF and VEGF receptors may indicate potential paracrine/autocrine roles of VEGF in granulosa cells. The following effects of VEGF on granulosa cell function have been described.

Cell proliferation. Reports on the proliferative effects of VEGF on granulosa cells are conflicting. In their study, Greenaway and co-workers did not find any effect of VEGF treatment on bovine granulosa cell proliferation in follicles of any size (Greenaway, Connor, Pedersen et al. 2004). In contrast, Grasselli et al (2002) demonstrated that VEGF (0.026 to 1.3 nmol/l) dose-dependently stimulated proliferation of porcine granulosa cells from large (>6 mm) but not small (<3 mm) follicles (Grasselli, Basini, Bussolati et al. 2002), with doses of 0.26 to 0.52 nmol/l maximally stimulating proliferation.

Cell survival. The first suggestion that VEGF may be involved in granulosa cell survival came from a study by Quintana et al (2001) who examined the relationship between VEGF production and apoptosis in cultures of human granulosa cells. This group found that VEGF concentrations in supernatants from cultures of granulosa cells from patients with decreased response to ovarian hyperstimulation were low whereas apoptosis in those cultures was high (Quintana, Kopcow, Marconi et al. 2001). The opposite effect was observed when examining cultures of granulosa cells from patients with normal or enhanced response to ovarian hyperstimulation (Quintana, Kopcow, Marconi et al 2001). Consistent with these findings, Greenaway et al (2004) demonstrated that VEGF reduced the induction of active caspase-3, a mediator of apoptosis, by tumour necrosis factor α (TNFα) in cultures of bovine

granulosa cells (Greenaway, Connor, Pedersen et al. 2004). The effects of VEGF were reversed by addition of a specific inhibitor of VEGFR2 (SU1498). These results indicated that VEGF and VEGFR2 interact in a coordinated manner to protect granulosa cells against apoptosis. The findings of this study are in agreement with those of Kosaka et al (2007) who found that VEGF and FSH had similar effects on bovine granulosa cell survival. They proposed that VEGF was capable of inhibiting apoptosis in granulosa cells possibly through effects on the Akt or ERK signalling pathways (Kosaka, Sudo, Miyamoto et al. 2007). Similarly, a study in rats involving inhibition of VEGF by in vivo administration of VEGFR1 peptide resulted in an increase in granulosa cell apoptosis due to an imbalance between antiapoptotic and proapoptotic signals (Abramovich, Parborell, & Tesone 2006). Consistent with this, treatment of rat granulosa cells with VEGF before cryopreservation has been shown to reduce the damage induced by the freeze-thaw process (Shin, Lee, Lee et al. 2006), once again providing evidence that VEGF functions to protect against granulosa cell apoptosis.

1.3. Signalling and crosstalk mechanisms of VEGF and heterotrimeric G-protein pathways

1.3.1 VEGFR Signalling

VEGFRs belong to the receptor tyrosine kinase (RTK) superfamily of cell surface receptors which have intrinsic protein tyrosine kinase activity. This involves the transfer of a phosphate molecule from a donor ATP molecule to the hydroxyl groups of tyrosine amino acids on target proteins (Hunter 1998). RTKs have been shown to be key regulators in normal cell processes, including proliferation, differentiation, survival, migration, progression through the cell cycle and metabolic activity (Schlessinger 2000) and in addition have been found to have a critical role in the development and progression of many types of cancers (Zwick, Bange, & Ullrich 2001). Some 58 RTKs have been grouped in 20 subfamilies based on their kinase domain sequence (Robinson, Wu, & Lin 2000).

1.3.2 Activation of VEGF receptors

Binding of ligand to extracellular domains of RTKs induces changes in conformation leading to receptor dimerization. It has been demonstrated that VEGFR dimerization is critical for signal transduction (Fuh, Li, Crowley et al. 1998). Dimerisation of RTKs acts to stabilise interactions between adjacent cytoplasmic domains and to stimulate receptor tyrosine kinase activity, resulting in phosphorylation of specific tyrosine residues in the intracellular receptor domains (autophosphorylation) which are targets for (SH2) and phosphotyrosine binding (PTB) domain-containing proteins, including phospholipase Cγ (PLCγ), phosphoinositide 3 kinase (PI3K) and Src (Reviewed by Ullrich & Schlessinger 1990; Zwick, Bange, & Ullrich 2001). Signalling pathways putatively mediating the effects of ligand-induced activation of VEGFR2 are shown schematically in Figure 1.7.

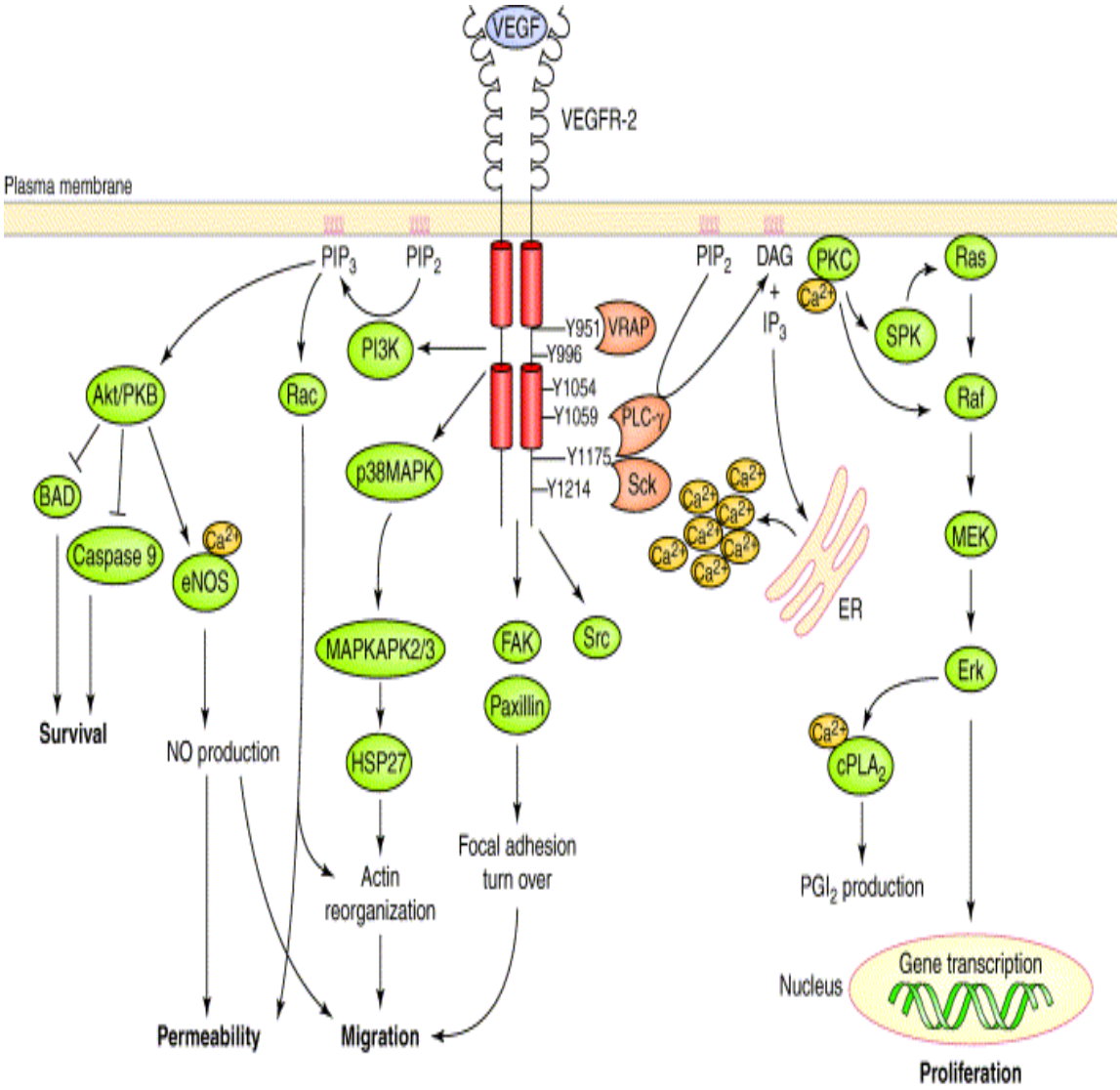


Figure 1.7. Schematic diagram of the signalling pathways putatively mediating the effects of activation of VEGFR2 in endothelial cells. Adapted from (Cross, Dixelius, Matsumoto et al. 2003) and (Takahashi & Shibuya 2005).

In contrast to VEGFR2, the signalling ability of VEGFR1 remains controversial. VEGFR1 is considered to be a kinase-impaired RTK because it is poorly phosphorylated after ligand binding and its ability to phosphorylate RTK substrates is negligible. A recent study by Meyer et al (2006) has confirmed that this characteristic of VEGFR1 is due to a single amino acid substitution (Asp → Asn) in the activation loop that exists on the intracellular kinase domain of the receptor (Meyer, Mohammadi, & Rahimi 2006). These authors propose that the conserved

aspartic acid in RTKs favours transphosphorylation of this activation loop which is necessary for receptor activity.

1.3.3 Activation of PLCγ and ERK1/2 by VEGFR2

PLCγ Pathway. PLCγ is a substrate for most RTKs. To date, two mammalian PLCγ isoforms have been identified, PLC-γ1 and PLCγ-2 (Suh, Park, Manzoli et al. 2008). PLC-γ1 is the best characterised isoform and from here will be referred to as PLCγ. All PLC isoforms share a catalytic domain composed of two regions of high homology (40-60%), X and Y. The region between X and Y contains Src homology domain 2 (SH2) and Src homology domain 3 (SH3) that are unique to PLCγ (Reviewed by Rhee & Bae 1997). The presence of these domains aids the association of PLCγ with other proteins, where SH2 recognises phosphotyrosine sequences and SH3 recognises proline-rich sequences (Carpenter & Ji 1999). Following VEGFR2 activation, phosphorylation at Y1175 allows the binding, phosphorylation and subsequent activation of PLCγ (Takahashi, Yamaguchi, Chida et al. 2001). PLC activation results in hydrolysis of membrane-bound phosphatidylinositol 4,5-biphosphate (PIP2) into the second messengers, inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 rapidly diffuses through the cytoplasm to activate IP_3 receptors on the smooth endoplasmic reticulum membrane resulting in the opening of IP_3-gated-calcium (Ca^{2+}) channels on the endoplasmic reticulum The increase in intracellular (Ca^{2+}) results in activation of calcium-dependent kinases, such as calmodulin, and other membrane-bound, cytosolic and mitochondrial proteins (Reviewed by Balla 2006). After PLC-mediated PIP2 cleavage, DAG remains on the cell membrane where it can activate members of the protein kinase C (PKC) enzyme family (Nishizuka 1988) as well as a variety of non-PKC effectors (Yang & Kazanietz 2003). Many PKCs are calcium dependent and it is believed that the increase in Ca^{2+} concentration induced by IP_3 causes PKC to translocate from the cytoplasm to the plasma membrane. PKC is anchored to the cell membrane via receptors for activated PKC (RACK) before it is activated by DAG, Ca^{2+} and phosphatidylserine. Both IP_3- and DAG-induced signals eventually target a range of

transcription factors that are responsible for the effects of PLC activation on cell functions (Reviewed in Rebecchi & Pentyala 2000).

MAPK Pathway. One of the putative targets of PLCγ/PKC is the ERK1/2 signalling cascade. This is a classical RTK-activated cascade that is involved in regulation of cell survival, proliferation, differentiation and motility (Reviewed in Rebecchi & Pentyala 2000). RTKs classically activate ERK1/2 through growth factor receptor-bound protein 2 (Grb2) and guanine nucleotide exchange factor, Sos (son of sevenless) targeting of membrane-bound Ras (McCormick 1993) although the use of this mechanism by VEGFR2 is controversial. Recent studies have shown that VEGF can activate Ras through a pathway that requires PKC and sphingosine kinase (Meadows, Bryant, & Pumiglia 2001; Shu, Wu, Mosteller et al. 2002). Raf, a serine/threonine specific kinase, is subsequently phosphorylated and activates MEK protein kinases which in turn phosphorylate and activate the serine/threonine specific protein kinases, ERK 1 and 2. Activated ERK1/2 translocates to the nucleus where it phosphorylates target transcription factors to induce changes in gene expression.

1.3.4 Heterotrimeric G-protein Signalling

Heterotrimeric G-proteins are involved in the transduction of signals from heptahelical transmembrane receptors, known as G-protein coupled receptors (GPCRs), the largest family of cell surface receptors with more than 1000 encoding genes that relay signals from a variety of ligands such as hormones, neurotransmitters, and chemokines in addition to autocrine and paracrine factors to regulate a wide range of cellular responses including sight, smell, neuronal activity and cell proliferation, survival and migration (Rens-Domiano & Hamm 1995).

1.3.5 Heterotrimeric G-Protein subunits and classification

Heterotrimeric G-proteins are composed of three subunits, α subunit, with GTPase activity, and β and γ subunits that are usually found in the cell tightly associated as Gβγ dimers (Morris & Malbon 1999). Different types of each subunit (21 different

Gα subunits, 6 Gβ subunits and 12 Gγ subunits) have been described (Oldham & Hamm 2008).

A given type of Gα can trimerise with any of multiple combinations of Gβγ and therefore G-protein families are classified according to the specific Gα subunit into Gs, Gq, Gi/o and G12/13 (see below). However, the emerging importance of the Gβγ complex have led to suggestions that the nomenclature should be revised (Morris & Malbon 1999).

Gα subunits are 39–52 kDa peptides with approximately 45-80% shared homology (Simon, Strathmann, & Gautam 1991). Each Gβ subunit has a molecular mass of approximately 350 kDa and each Gγ is approximately 7-9 kDa in size. Gβ1-4 form a tight complex with Gγ which can only be broken under denaturing conditions (Morris & Malbon 1999). The Gβ5 subunit is an exception as its association with Gγ is considerable weaker than other Gβ subunits. There are is a large number of different Gβγ complexes resulting from all possible subunit combinations. The roles of Gβγ in cell signalling have been less well characterised than those of Gα subunits. The function of Gβγ complexes was first thought to be restricted to facilitation of coupling of Gαβγ trimers to activated GPCRs and inhibition of Gα activity through the intrinsic GTAPase activity of Gβγ (McCudden, Hains, Kimple et al. 2005). However it is now clear that Gβγ dimers themselves can activate a variety of downstream effectors that include PLCs (Rhee & Bae 1997), ACs (Sunahara, Dessauer, & Gilman 1996), ion channels (Schneider, Igelmund, & Hescheler 1997), PI3K (Vanhaesebroeck, Leevers, Panayotou et al. 1997; Zaballos, Garcia, & Santisteban 2008) and ERK (Lopez-Ilasaca 1998; Reusch, Schaefer, Plum et al. 2001).

Gαs Class. This class contains two members, Gαs and Gαolf, which activate the cAMP-producing enzyme, adenylyl cyclase (AC). Cyclic AMP activates Protein kinase A (PKA) as well as Exchange proteins directly activated by cAMP (EPAC) (Reviewed by Bos 2006). In addition to AC, Gαs can also directly stimulate the Src family of tyrosine kinases (Ma, Huang, Ali et al. 2000). Gαs subunits are substrates for ADP-ribosylation by cholera toxin, which prevents exchange of GTP for GDP thus maintaining the protein in the activated state (Kahn & Gilman 1984).

Gαq Class. The Gαq class includes Gα11 and Gαq which are ubiquitous in mammalian tissues. Other members of this class include Gα14, Gα15 and Gα16, the expression of which are limited to stromal, epithelial and hematopoietic cell types (Morris & Malbon 1999). Activating GPCRs do not appear to discriminate between Gα11 and Gαq (Wange, Smrcka, Sternweis et al. 1991). Members of the Gαq class can activate effectors including phospholipase Cβ (PLCβ) (Dowal, Provitera, & Scarlata 2006), Rho-specific guanine nucleotide exchange factors (GEFs) (Rojas, Yohe, Gershburg et al. 2007) and Bruton's tyrosine kinase (BTK) (Bence, Ma, Kozasa et al. 1997). Recently, a Gq/11-specific inhibitor has been developed (YM-254890) which blocks the exchange of GDP for GTP by Gαq/11 (Takasaki, Saito, Taniguchi et al. 2004).

Gαi/o Class. The Gi class consists of Gαi1, Gαi2, Gαi3, Gαo1, Gαo2, Gαz and Gαt. Whereas Gαi1-3 are ubiquitous, Gαo1-2 are specific to the brain and Gαt is specific to the retina In contrast to Gαs, Gαi inhibits AC activity (Reviewed by Morris & Malbon 1999). Gαi/o family members have also been found to activate the ERK signalling cascade via activation of c-Src/STAT-3 and RAP pathways (Weissman, Ma, Essex et al. 2004). Pertussis toxin (PTX) ATP-ribosylates the COOH-terminal cysteinyl residue of Gαi/o, thus uncoupling the G protein from its activating receptor (Reviewed by Morris & Malbon 1999). All members of the Gαi/o class with the exception of Gαz are sensitive to PTX.

Gα12/13 Class. Gα12 and Gα13 are expressed ubiquitously and are often activated by receptors that also couple to Gq/11 (Dhanasekaran & Dermott 1996). Since specific molecular inhibitors of this class are not available relatively little is known about their function. Over-expression studies have shown that Gα12 and Gα13 can interact with a variety of effectors including Rho (Buhl, Johnson, Dhanasekaran et al. 1995), BTK (Jiang, Ma, Wan et al. 1998) and HCLS1-associated protein X-1 (HAX-1) (Radhika, Onesime, Ha et al. 2004).

1.3.6 Heterotrimeric G-Protein Activation

In the inactive state, the GDP-bound Gα subunit and Gβγ dimer are associated as a trimer in the inner face of the cell membrane. When an activated GPCR interacts with the G-protein trimer, the GDP on the Gα subunit is exchanged for GTP (figure 1.8). This results in the dissociation of the Gα subunit from the Gβγ complex. Both Gα and Gβγ are released separately to stimulate a number of downstream effectors. G-protein signalling is terminated by the hydrolysis of GTP by GTPase activity intrinsic to the Gα subunit. The resultant GDP-bound Gα subunit subsequently re-associates with Gβγ dimer to re-establish a trimer ready to enter another activation cycle in the presence of an activated GPCR. More detailed reviews on the mechanism of G-protein activation can be found elsewhere (Cabrera-Vera, Vanhauwe, Thomas et al. 2003; Oldham & Hamm 2008; Wettschureck & Offermanns 2005). There are intracellular modulators of heterotrimeric G protein activity in addition to GPCRs. These modulators act to inhibit or promote the activated state of G proteins to ensure that their response to GPCR activation is limited in time and space. These modulators include heterotrimeric G protein effectors themselves. For example, all PLCβ isoforms stimulate the GTPase activity of the Gα subunit to accelerate the hydrolysis of GTP to GDP thus rendering the G protein inactive (Rebecchi & Pentyala 2000). A further level of control is provided by regulators of G-protein signalling (RGS), a protein family with more than thirty members that specifically interact with Gα subunits to enhance their Gα intrinsic GTPase activity (Reviewed by De, Zheng, Fischer et al. 2000; Hollinger & Hepler 2002). There are also guanine nucleotide exchange factors (GEFs) that promote GDP to GTP exchange on Gα subunits to increase heterotrimeric G protein activity (Hagemann, Narzinski, & Baranski 2007). In addition, a variety of scaffolding proteins are known to interact with and provide spatial regulation of heterotrimeric G proteins (Reviewed in Andreeva, Kutuzov, & Voyno-Yasenetskaya 2007). Scaffolding proteins have been defined as proteins that associate with two or more partners to enhance the efficiency and/or specificity of cellular signalling pathways (Hall & Lefkowitz 2002). These proteins function as adaptors or scaffolds to selectively bridge the -N or -C terminal or other regions of RGS proteins to GPCRs,

G-proteins or effectors. Scaffolding proteins can strengthen, modify or convey additional selectivity to RGS proteins (Hall & Lefkowitz 2002).

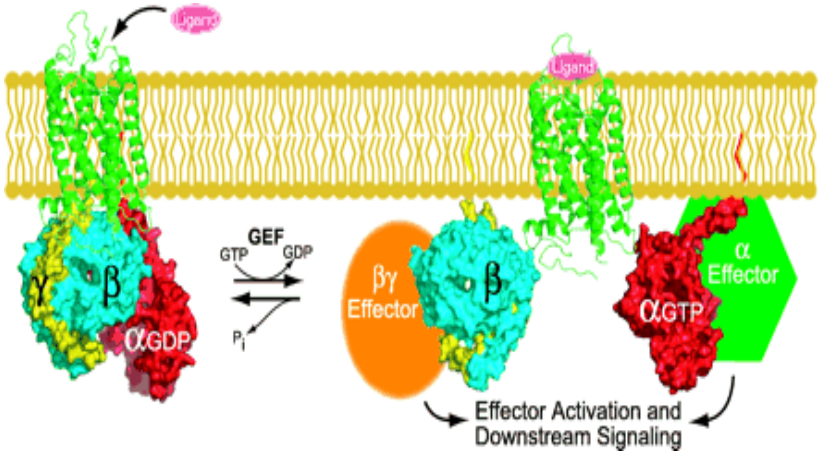


Figure 1.8. Illustration of heterotrimeric G-protein activation at the plasma membrane. An activated GPCR acts as a guanine-nucleotide exchange factor to promote GDP for GTP exchange resulting in dissociation of Gα subunit from Gβγ complex and subsequent activation of downstream effectors. G-protein signalling is terminated by hydrolysis of GTP resulting in re-association of GDP-bound Gα subunit with Gβγ dimer. Taken from (Jones, Siderovski, & Hooks 2004)

1.3.7 Heterotrimeric G-Protein activation of PLCβ

Four mammalian PLCβ isoforms have been identified (PLCβ1, PLCβ2, PLCβ3 and PLCβ4). The most widely expressed isoform is PLCβ-1, which has particularly high activity in certain areas of the brain (Reviewed by Rebecchi & Pentyala 2000). All isoforms are soluble in water but bind strongly to inner surface of the cell membrane with little specificity (Runnels, Jenco, Morris et al. 1996). Evidence also exists for a nuclear PLCβ system, which functions independently of the cell membrane system (Reviewed by Cocco, Martelli, Mazzotti et al. 2000). Nuclear expression is highest for PLCβ-1 followed by PLCβ-3, PLCβ-2 and PLCβ-4 (Cocco, Martelli, Mazzotti er al. 2000).

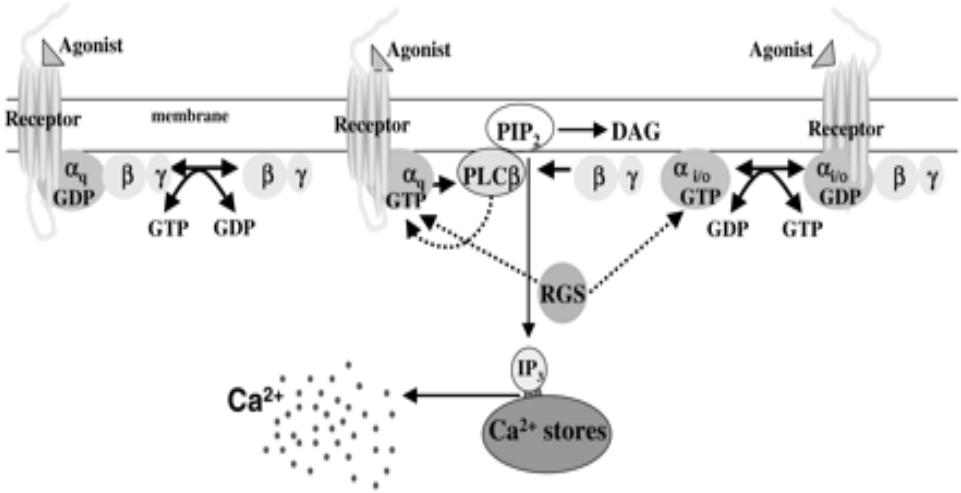


Figure 1.9. Both Gαq/11 and Gβγ (putatively derived predominantly from Gi within many cells) can activate PLCβ isoforms. PLCβ. PLCβ catalyses the hydrolysis of PIP_2 in the cell membrane to the second messengers, IP_3 and DAG, which subsequently induce the release of intracellular calcium and the activation of PKC. RGSs act to down modulate the activity of Gα subunits. Taken from (Rebecchi & Pentyala 2000).

Both Gq/11 (through Gαq/11) and Gi (through Gβγ) are recognised as putative activators of PLCβ isoforms (see figure 1.9). All Gα subunits of the Gq/11 family stimulate PLCβ but not PLCγ or PLCδ (Berridge 1993; Exton 1996; Rhee & Choi 1992). However, PLCβs are differentially sensitive to Gαq subunits in a rank order of PLCβ1 > PLCβ3 > PLCβ2 (Rhee & Bae 1997). Furthermore, PLCβ4 isoform has been reported to be insensitive to Gαq/11 stimulation (Kim, Min, Ryu et al. 1998). However, experiments involving purified PLCβ1, PLCβ2 and PLCβ3 have demonstrated that there is little difference between the activation of PLCβ isoforms by purified members of the Gαq/11 family (Hepler, Kozasa, Smrcka et al. 1993; Kozasa, Hepler, Smrcka et al. 1993; Smrcka & Sternweis 1993), therefore the physiological role of Gαq/11 in activating different PLCβ subunits needs to be clarified.

In contrast to Gαq subunits, Gβγ can activate all PLCβ isoforms. The sensitivity of PLCβ to Gβγ dimers differs in a rank order of PLCβ3 > PLCβ2 > PLCβ1 > PLCβ4 (Park, Jhon, Lee et al. 1993). Although it has not been possible to test every known Gβγ combination, those that have been investigated appear to be completely

interchangeable when reconstituted with PLC in artificial vesicles (Boyer, Graber, Waldo et al. 1994) or when co-expressed in living cells (Watson, Katz, & Simon 1994). In general, Gαq subunits are 50-100 fold more potent activators of PLCβ isoforms than Gβγ subunits (Morris & Malbon 1999). PLCβ3 is an exception whereby Gαq and Gβγ subunits have similar activating capacities on a stoichiometric basis (Rebecchi & Pentyala 2000).

1.3.8 Cross-Talk between RTK and heterotrimeric G-protein pathways

1.3.8.1 GPCR transactivation of RTKs

Transactivation of RTKs by GPCR ligands is a well-described phenomenon (Luttrell, Daaka, & Lefkowitz 1999; Shah & Catt 2004; Werry, Sexton, & Christopoulos 2005; Wetzker & Bohmer 2003). In the ovary, LH stimulation of granulosa cells activates the Epidermal Growth Factor (EGF) signalling system during the late periovulatory period (Reviewed by Conti, Hsieh, Park et al. 2006). Following LH/hCG stimulation, mRNA levels for EGF-like growth factors, epiregulin (EREG), amphiregulin (AREG) and betacellulin (BTC), increase both in vitro and in vivo (Espey & Richards 2002; Park, Su, Ariga et al. 2004; Sekiguchi, Mizutani, Yamada et al. 2004). These factors have been shown to induce oocyte maturation as efficiently as LH (Conti, Hsieh, Park, & Su 2006). It is believed that these factors exert their effects on the oocyte via the cumulus cells because denuded oocytes did not undergo maturation in the presence of these growth factors (Conti, Hsieh, Park, & Su 2006). In support of these findings, when EGFR inhibitor was injected directly into the ovarian bursa of the rat, there was a 50% decrease in ovulation and many follicles were observed to have trapped oocytes present in comparison with the contralateral ovary that was injected with a saline control. In addition, mice that are deficient in either amphiregulin or epiregulin display either delayed or reduced oocyte maturation (Hsieh, Lee, Panigone et al. 2007). The LH surge is believed to induce EGF-like growth factor shedding from the cell membrane through activation of matrix metalloproteinases (MMPs) (Park, Su, Ariga et al. 2004). This was demonstrated by studies involving MMP inhibition which resulted

in blocked oocyte maturation (Ashkenazi, Cao, Motola et al. 2005). A recent study by Downs and Chen (2008) has demonstrated that the FSH receptor can also transactivate the EGF network (Downs & Chen 2008). These authors suggest that EGF-like peptides are released from cumulus cells in response to stimulation with FSH and that these factors have a role at the local level to regulate meiosis (Downs & Chen 2008).

1.3.8.2 RTK activation of heterotrimeric G-protein pathways

Another mechanism of crosstalk between heterotrimeric G-proteins and RTKs is exemplified by the reported involvement of heterotrimeric G-proteins in RTK signalling. This has been demonstrated in a number of examples (Reviewed by Patel 2004) as follows:

EGFR. The activation of PLC by EGF in rat whole liver has been shown to involve Gi/o family members as demonstrated by the inability of EGF to increase intracellular Ca^{2+} in the presence of pertussis toxin (PTX) (Liang & Garrison 1991; Rashed & Patel 1991). Further support for a role of G-proteins in the activation of EGF was provided by Nair et al (1990) who showed that Gs activity in isolated rat hearts was involved in stimulation of the PLC pathway (Nair, Parikh, Milligan et al. 1990). Interestingly, a short (13 amino acid) Gs-activating sequence has been identified in the cytoplasmic region of the EGF receptor (Sun, Chen, Poppleton et al. 1997; Sun, Seyer, & Patel 1995). Further, an interaction between Gs and this activating region on EGF receptor has been reported necessary for efficient phosphorylation of this G-protein (Poppleton, Sun, Mullenix et al. 2000).

Insulin receptor (IR). The first evidence that the insulin receptor was functionally coupled to heterotrimeric G-proteins was presented in the mid 1980s (Goren, Northup, & Hollenberg 1985). In that study Goren and associates demonstrated that the ability of insulin to inhibit lypolysis and glucose oxidation in rat adipocytes was blocked by exposure to Pertussis toxin. Consequently there were reports that showed that the insulin receptor couples to members of the Gαi/o family (Ciaraldi & Maisel

1989; Krupinski, Rajaram, Lakonishok et al. 1988; Rothenberg & Kahn 1988). It was further elucidated that Gαi2 was specifically involved in insulin-mediated signalling (Moxham & Malbon 1996; Song, Zheng, Malbon et al. 2001; Zheng, Guo, Wang et al. 1998). Furthermore, Gαq/11 was also demonstrated to associate with the insulin receptor and to be phosphorylated in the presence of insulin (Imamura, Vollenweider, Egawa et al. 1999). Additionally, Gαq/11 was found to stimulate insulin-mediated translocation of GLUT4, an insulin-regulated glucose transport molecule, to the cell membrane (Imamura, Vollenweider, Egawa et al. 1999). These results indicate that Gαq/11 has a role in glucose transport in cells. There have also been a number of reports to suggest that the actions of IGF are mediated by heterotrimeric G-proteins (Reviewed by Patel 2004).

Platelet-Derived Growth Factor receptor (PDGFR). The PDGF-stimulated phosphorylation of ERK has been reported to involve Gβγ subunits that are likely derived from Gi protein as this effect can be inhibited by pertussis toxin (Conway, Rakhit, Pyne et al. 1999). It has been shown in HEK cells that PDGFR and endothelial differentiation gene 1 (EDG1, a GPCR that is activated by sphingosine-1 phosphate) form a tethered complex. This complex has been shown to provide a platform on which receptor crosstalk can occur to stimulate more efficient regulation of downstream pathways such as ERK activation (Alderton, Rakhit, Kong et al. 2001).

Fibroblast Growth Factor receptor (FGFR). Very little is known about the interaction of FGF with heterotrimeric G-proteins although one study demonstrated that upon FGF binding to FGFR, AC was stimulated whilst NADPH-dependent H_2O_2 production was inhibited in adipose cell membranes (Krieger-Brauer, Medda, & Kather 2000). Krieger-Brauer et al (2000) confirmed that these effects were confined to the cell membrane and occurred without ATP, indicating that classical RTK activity of the FGFR was not required. The stimulatory effect of FGF on AC could be blocked by an Anti Gs peptide, thus showing that FGF could associate with Gs. G-protein activity was also required for inhibition of NADPH-dependent H_2O_2

synthesis as indicated by the ability of a Gβγ-sequestering fragment to reverse this effect.

In addition to RTKs, G-proteins have been shown to associate with a variety of non-classical receptor types, for example, zona pellucida protein 3 (ZP3), a receptor that is involved in fertilisation. ZP3, contains O-linked oligosaccharide ligands which are responsible for binding to β-1,4 galactosyltransferase (GalTase) that is present on the sperm (Wassarman 1999). When sperm and egg meet, ZP3 and GalTase interact and Gi is subsequently activated which in turn initiates the acrosome reaction (Gong, Dubois, Miller et al. 1995). Other examples of non-GPCR family receptors functionally coupled to G-proteins include integrins, T cell receptors and natriuretic peptide receptors (Reviewed by Patel 2004).

1.3.9 Crosstalk between VEGF Receptor and heterotrimeric G-protein pathways

Zeng and associates demonstrated that VEGFR2 but not VEGFR1 was involved in VEGF-induced endothelial cell proliferation and migration (Zeng, Dvorak, & Mukhopadhyay 2001a; Zeng, Zhao, & Mukhopadhyay 2002a). In fact, VEGFR1 had an inhibitory effect on endothelial cell proliferation (Zeng, Zhao, & Mukhopadhyay 2002a). This down-regulation of endothelial cell proliferation occurred through the PI3K pathway as shown by a reversal on the inhibitory effect on proliferation by treatment with PI3K inhibitors (Zeng, Dvorak, & Mukhopadhyay 2001a). To identify whether heterotrimeric G-proteins were involved in VEGF-induced proliferation and migration, these authors first investigated whether the canonical G-Protein/PLCβ pathway was activated. It is generally accepted that stimulation of a VEGF-responsive cell normally results in induction of PLCγ phosphorylation within 5 minutes, as observed by an intracellular rise in calcium (Ca^{2+}) concentration (Zeng, Sanyal, & Mukhopadhyay 2001). However, Mukhopadhyay and associates (2004) found that following treatment of endothelial cells with VEGF there was an accelerated rise in intracellular Ca^{2+} after only 40 seconds, thus suggesting that phosphorylation of PLCγ may not be responsible for the increased Ca^{2+}

concentration (Mukhopadhyay, Zeng, & Bhattacharya 2004). They proposed that the increased Ca^{2+} levels may be mediated instead by PLCβ. This was tested in a study that measured the catalytic activities of PLCβ isoforms following VEGF stimulation and found that PLCβ3 and not PLCγ was activated by VEGF stimulation. The authors then tested the involvement of heterotrimeric G-proteins in the VEGF-induced Ca^{2+} increases and found no effects of PTX on such response. Leading to the conclusion that Gαi/Gαo were not involved. Using a Gα11 antagonist fusion peptide Mukhopadhyay et al (2004) found that the VEGF-stimulated increase in Ca^{2+} was instead predominantly mediated by the Gα11/PLCβ3 signalling pathway. This was further confirmed by inhibition of Gαq, which severely impaired PLCβ-3 activation (Reviewed by Mukhopadhyay, Zeng, & Bhattacharya 2004).

Further evidence for the involvement of G-proteins in VEGF signalling comes from studies in the zebrafish. It has been shown that targeted knockdown of the G protein *GNG2* (Gγ2) leads to a block in normal angiogenesis in the zebrafish embryo (Leung, Chen, Stauffer et al. 2006). Loss of *GNG2* reduced the ability of VEGF to promote the angiogenic sprouting of blood vessels by disrupting VEGF induced phosphorylation of PLCγ1 and serine/threonine kinase AKT was compromised (Leung, Chen, Stauffer et al. 2006). *GNG2* was not necessary for VEGFR2 activation indicating that, in contrast to what had been previously reported for G11 by Zeng and associates (2003) the involvement of *GNG2* is not likely to occur at the level of the receptor, but downstream in the VEGFR signalling cascade (Leung, Chen, Stauffer et al 2006; Zeng, Zhao, Yang et al. 2003). Another recent study found that Gα13 is required for migration of fibroblast and endothelial cell types (Shan, Chen, Wang et al. 2006), Furthermore, mouse cells that were treated with Gα13 siRNA had reduced levels of VEGF-induced endothelial cell migration (Shan, Chen, Wang et al. 2006).

1.4 **Overall Aims**

From the afore-reviewed literature, it is clear that there is a paucity of information on the roles of VEGF in follicle development, particularly those roles involving non-vascular follicle components. Based on above-presented information on other cell types, such roles, if confirmed, may very well involve critical interactions at the intracellular level with heterotrimeric G-protein pathways, most notably, gonadotrophin-activated pathways.

A better understanding of the roles of VEGF during follicle development will not only expand our knowledge on the basic mechanisms of follicle development but may also aid in the design of therapeutic approaches to better control reproductive cycles in animals and humans alike.

Consequently, the aims of the work described in this thesis were:

1. To investigate the roles of VEGF in granulosa cell function during the development of bovine dominant follicles
2. To investigate the involvement of heterotrimeric G-protein pathways in such roles of VEGF in dominant follicles

CHAPTER 2:

Roles of VEGF on bovine granulosa cell function.

2.1 Abstract.

The current study used an *in vitro* bovine granulosa cell model to investigate the roles of VEGF in specific granulosa cell functions during the development of dominant follicles. Granulosa cells were obtained from healthy follicles with diameters of 4 to 8 mm (corresponding to just before the selection of a dominant follicle during a follicular wave) or 9 to 14 mm (encompassing all developmental stages of a dominant follicle) and exposed to a range of VEGF concentrations (1 to 100 ng/ml) encompassing concentrations found naturally in bovine dominant follicles. A concentration of VEGF of 1 ng/ml induced significant proliferation of bovine granulosa cells from 4 to 8 mm follicles ($P = 0.024$) and increased the proliferative response of these cells to FSH ($P = 0.045$), whereas higher doses of VEGF had no effect on proliferation ($P > 0.1$). None of the concentrations of VEGF, alone or in combination with FSH, had an effect on expression of the steroidogenic enzyme, *CYP11A1,* by these cells ($P > 0.1$). In contrast, VEGF induced a dose-dependent increase in ERK1/2 activation by granulosa cells from 4 to 8 mm follicles ($P < 0.03$). Granulosa cells from 9 to 14 mm follicles responded to 1 ng/ml VEGF with an increase in *COX-2* ($P = 0.003$) but higher doses had no effect ($P > 0.1$) Treatment with 1 ng/ml VEGF, however, abolished an LH-induced increase ($P < 0.0002$) in *COX-2* in these cells. In addition specific inhibition of VEGFR2 prevented ($P > 0.1$) LH stimulation of both ERK phosphorylation and *COX-2* expression in granulosa cells from 9 to 14 mm follicles. These results suggest important roles of VEGF in granulosa cell function during the development of the dominant follicle. VEGF may act, at least partially, by mediating the effects of gonadotrophins on granulosa cells.

2.2 Introduction

Follicle development in monovular species involves the synchronous growth of antral follicles as follicular waves. Within each wave, usually one follicle (dominant follicle) is selected to undergo pre-ovulatory maturation whereas the remaining follicles become subordinate and eventually undergo atresia. In cattle, follicle dominance is established when the largest follicle of a wave reaches about 8.5 mm and this follicles continues to grow to about 14 to 20 mm when it normally ovulates or begins regressing (Ginther, Beg, Donadeu et al. 2003). The control of follicle selection and the growth of the dominant follicle lies within complex interactions between systemic gonadotrophins and local growth factors. For example, the synergistic actions of IGF-I and FSH are critical in the selection of a single follicle from the cohort of a wave (Mihm & Evans 2008) whereas EGF plays a very important role in mediating some of the responses of the preovulatory follicle to the ovulatory LH surge (Conti, Hsieh, Park, & Su 2006). The role of many other follicular factors remains poorly understood. One such factor is VEGF.

Numerous studies have shown that VEGF and its receptors (VEGFR1 and 2) are expressed in granulosa cells (Greenaway, Connor, Pedersen et al. 2004; Ravindranath, Little-Ihrig, Phillips et al. 1992; Barboni, Turriani, Galeati et al. 2000; Shin, Lee, Ko et al. 2005; Watson & Al-Zi'abi 2002; Gordon, Mesiano, Zaloudek et al. 1996). Dynamic changes in the expression of VEGF and its receptors have been reported throughout follicle development in primates (Taylor, Hillier, & Fraser 2004; Kamat, Brown, Manseau et al. 1995; Otani, Minami, Yamoto et al. 1999), pigs (Barboni, Turriani, Galeati et al. 2000; Shimizu, Kawahara, Abe et al. 2003; Mattioli, Barboni, Turriani et al. 2001) and cattle (Greenaway, Connor, Pedersen, Coomber, LaMarre, & Petrik 2004; Yang & Fortune 2007), with a consistent increase in both VEGF production and expression of its receptors in dominant follicles.

Studies in non-human primates have shown that intrafollicular or systemic injection of a VEGF antagonist results in significant impairment of ovulation and subsequent luteal function (Fraser, Wilson, Rudge et al. 2005; Hazzard, Xu, & Stouffer 2002). Further, Danforth et al (2003) showed that VEGF can stimulate pre-antral follicle development in culture and Shimizu et al (2007) recently demonstrated that intraovarian injection of VEGF promoted follicle development to the preovulatory stage in gilts.

The mechanisms by which VEGF elicits those effects in follicles are not yet fully understood. While the role of VEGF is undoubtedly associated, at least in part, with the induction of an extensive vascular system in the follicle, limited studies using ruminant or porcine cells suggest that VEGF may modulate granulosa cell function directly by promoting survival (Greenaway, Connor, Pedersen et al. 2004; Kosaka, Sudo, Miyamoto et al. 2007) and/or proliferation (Grasselli, Basini, Bussolati et al. 2002). VEGF has been shown to promote survival of a variety of other cell types including cells of the central nervous system (Sondell, Lundborg, & Kanje 1999a; Jin, Mao, & Greenberg 2000a; Jin, Mao, & Greenberg 2000b; Oosthuyse, Moons, Storkebaum et al. 2001), haematopoietic cells (Gerber, Malik, Solar et al. 2002) and tumour cells (Pidgeon, Barr, Harmey et al. 2001; Chung, Bachelder, Lipscomb et al. 2002; Barr, Pidgeon, & O'Byrne 2006). In addition, VEGF can induce proliferation of non angiogenic cells during bone formation (Gerber, Vu, Ryan et al. 1999), hematopoiesis (Ferrara, Carver-Moore, Chen et al. 1996), wound healing (Wilgus, Matthies, Radek et al. 2005) and neural development (Sondell, Lundborg, & Kanje 1999b; Zhu, Jin, Mao et al. 2003).

The observations that granulosa cells are a major site of VEGF synthesis within the follicle (Fraser & Wulff 2001) and that expression of both VEGF and its receptors increase in the dominant follicle (Ginther, Gastal, Gastal et al. 2004b) reaching their maximal levels in preovulatory follicles (Greenaway, Connor, Pedersen et al. 2004) suggest potentially important non-angiogenic roles of this growth factor during follicle selection and the development of the dominant follicle. The aim of this study was to investigate such role(s) of VEGF by examining its effects on proliferative,

steroidogenic (*CYP11A1* expression) and ovulation-associated (*COX-2* expression) responses of bovine granulosa cells in culture. Potential interactions between VEGF and gonadotrophins in these cells were also studied.

2.3 Materials and Methods

2.3.1 Collection and culture of granulosa cells.

Bovine ovaries were obtained from a local abattoir and transported to the laboratory at 38°C in Phosphate Buffered Saline (PBS) containing kanamycin (50 μg/ml; Sigma, Dorset, UK). Upon arrival at the laboratory, ovaries were briefly trimmed free of extraneous tissues and rinsed in 70% alcohol before being added to pre-warmed McCoys 5a media containing penicillin/streptomycin (10000 units/ml10mg/ml, Sigma, Dorset, UK). Follicles were dissected from ovarian stroma and their surface diameter was determined in 2 axes using a ruler. Follicles were were dissected from ovarian stroma and their diameter was measured with a ruler. Follicles were deemed healthy if they were well vascularised with transparent, amber-coloured follicular fluid without debris (Yang & Rajamahendran 2000). Follicles which were opaque/cloudy with little vasculature were considered atretic and were discarded. Granulosa cells were harvested by dissection of follicles into hemispheres within small Petri dishes containing McCoy's 5a media and blunt-ended forceps were used to gently scrape follicle walls to release granulosa cells. Granulosa cells from 4 to 8 mm or 9 to 14 mm follicles (corresponding to diameters of follicles before and after the beginning of dominance, respectively) were pooled and the resulting cell suspensions were transferred to 15ml pre-warmed McCoys 5a culture media and centrifuged at 1500 rpm for 5 minutes. Red blood cell lysis was carried by adding 1 ml of lysis buffer (10mM $KHCO_3$, 150mM NH_4Cl, 0.1mM EDTA (tri sodium), pH = 8.0) to the granulosa cell pellets for 1 minute at room temperature. Cells were then washed and centrifuged at 1500 rpm for 5 minutes and finally either cultured or processed for polymerase chain reaction (PCR) analysis of VEGF receptor. Cells were in all instances, cultured in a humidified atmosphere with 5% CO_2 at 37^OC in 12- or 96- well plates (Nuclon, Nunc, Denmark) that had been pre-

coated with Fibronectin (Sigma, Dorset UK). Cells were cultured under serum free conditions (Gutierrez, Campbell, & Webb 1997b) in McCoy's 5a with 0.02M HEPES, 3mM L-Glutamine, 0.1% BSA, 1ml/100ml Pen/Strep, and ITS (10ng/ml bovine insulin 2.5mg/L transferrin and 4μg/L sodium selenite). Cell numbers were determined using a haemocytometer and viability was assessed using trypan blue exclusion. Typical viability before plating ranged from 30 to 40%. In addition, during initial validation of the culture system, granulosa cell steroid secretion (oestradiol and progesterone) was measured and both the levels of steroid production and cell morphology were found to be similar to those described by Gutierrez et al 1997 (Gutierrez, Campbell, & Webb 1997b).

2.3.2 Proliferation Assay

Granulosa cells from 4 to 8 mm follicles were plated in 96-well plates at a density of 50 000 cells per well in a total volume of 100μl of media for 24 hours prior to addition of treatments. Treatments were prepared in sterile 96 well plates which were equilibrated for 1 hour in the incubator before addition to cells. During each experiment, the following treatments were applied in triplicate: recombinant human VEGF (R&D Systems Minneapolis, USA; 0, 1, 10, 100 ng/ml), FSH (National Hormone and Peptide Program; Torrance, California, USA; 10 ng/ml), FSH (10 ng/ml) in combination with VEGF (1 ng/ml), LH (National Hormone and Peptide Program; Torrance, California, USA; 10 ng/ml). Following 72 hour incubation with treatments and a single wash with pre-warmed culture media, cell proliferation was measured using Celltiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Southampton, UK), according to manufacture's instructions. This assay uses a MTS Tetrazolium compound that is bio-reduced by cells into a coloured formazan product that is soluble in culture medium. This bio-reduction takes place in metabolically active cells, thereby allowing comparative analysis of viable cell numbers. Changes in colour were measured using a Wallac 1420 Multilabel Counter (Wallac, Turku, Finland). To correct for background absorbance a triplicate set of control wells containing culture media only were included and their absorbance was subtracted from the absorbance values of all other samples.

2.3.3 Western blotting analyses of p-ERK1/2

Granulosa cell populations from 4 to 8 mm and 9 to 14mm follicles were plated at a density of 500, 000 cells per well in 12-well plates and allowed to settle for 24 hours. Cells were then starved in culture media without ITS for another 24 hours. After starvation cells were washed with the same media and incubated in duplicate wells for 20 minutes at 37^{O}C with VEGF (0.1, 1, 10, 100 ng/ml), EGF (Sigma, Dorset UK, 100ng/ml), LH (National Hormone and Peptide Program; Torrance, California, USA; 100 ng/ml) or media alone. In some experiments, cells were incubated for one hour with a selective VEGFR2 inhibitor, ZM323881 (final concentration 50μM, Merk Chemicals Ltd., Nottingham, UK) prior to addition of treatments. After incubation with treatments all cells were washed twice with ice cold PBS and lysed with 70μl of RIPA buffer (150 mm NaCl, 50 mm Tris, 1 mm EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM Na3VO4, and 1 mM NaF, pH 7.4) by gentle rocking at 4^{O}C for 45 minutes. Lysates were centrifuged for 5 minutes at 13,000 rpm to remove nuclei and cell debris. Protein lysates were stored at -20^{O}C until required for immunoblotting.

Protein lysates were assayed for protein content using the DC protein assay kit (Bio-Rad Laboratories Inc. Hercules, CA). Twenty micrograms of protein from each lysate were heat-denatured in buffer containing 12% SDS, 40% glycerol, 30% β-mercaptoethanol, 300mM DTT, 120mM EDTA, 1mg/ml bromophenol blue, 375 mM TrisCl, pH 6.8 for 5 minutes at 100^{O}C before being separated on a 12% SDS-Polyacrylamide gel that was run at 150 volts for approximately 45 minutes (or until tracking dye neared bottom of plates). Prior to transfer, filter paper was soaked in transfer buffer (25nM Tris, 192mM glycine, 20% methanol) at 4^{O}C and a PVDF membrane was activated in 100% methanol for 15 seconds and then placed into transfer buffer. After running SDS-PAGE, the gel was immediately equilibrated in transfer buffer for 10 minutes. Proteins were then transferred onto PDVF membranes

using the semi-dry blotting apparatus, Trans-Blot SD (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), at 10 volts/80mA for 70 minutes at room temperature.

Following transfer, membranes were blocked for 2 hours in a solution (10mM Tris, 100mM NaCl, 0.2% Tween-20) containing 5% BSA and incubated overnight at room temperature with Phospho-p44/42 MAP Kinase antibody (Cell Signalling Technology Inc., Beverly, MA) diluted 1:3000 in the same solution but with 1% BSA. After primary antibody incubation, membranes were washed (5 times, 5 minutes per wash) with Tris Buffered Saline (TBS). Membranes were incubated with Horseradish Peroxidase (HRP)-conjugated anti-rabbit anti-immunoglobulin (1:10,000; Amersham Biosciences, Buckinghamshire, UK) for 1 hour at room temperature with gentle rotation. Membranes were then washed in Nonidet p-40-Tris Buffered Saline (N-TBS) for 10 minutes, placed in a new container and washed with TBS for 5 minutes. These wash steps were repeated once before immune complexes were visualised using Super Signal West Femto maximum sensitivity detection system (Pierce Chemical Inc., Rockford, IL) and imaged using Fluro-S Scanning System (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Densitometry analyses were done using Quantity One Analysis Software (Bio-Rad).

2.3.4 QPCR analyses of *CYP11A1* and *COX-2*

Granulosa cells were plated in 12-well plates at a density of 500, 000 cells per well and allowed to settle for 24 hours. Cells were then incubated in duplicate wells with media alone, FSH (10ng/ml) or LH (100ng/ml), VEGF (1, 10 or 100 ng/ml) or with a combination of FSH (10ng/ml) or LH (10ng/ml) and VEGF (1ng/ml). In some experiments, granulosa cells were pre-incubated with VEGFR2 inhibitor (ZM323881) for 1 hour prior to addition of treatments.

After a 48 h-incubation, total RNA was extracted using RNeasy Mini Kit (Quiagen, Sussex, UK). Cells were lysed by addition of 350µl of buffer RLT (containing β-Mercaptoethanol, 1%) and homogenised by passage through a 20-gauge needle 10 times. In brief, the RNA purification procedure is based on silica-gel membrane

technology where total RNA is adsorbed to the silica gel membrane in the presence of chaotropic salts which remove water from hydrated molecules in solution. Polysaccharides and proteins are not adsorbed and are removed. After washing, RNA is eluted in small volumes under low salt conditions. RNA concentration and quality were checked using a NanoDrop (ND-1000) spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). Total RNA was stored at -80^{O}C until cDNA synthesis.

First strand cDNA synthesis was conducted by incubating 500ng of Random Primers (Promega, Madison, WI, USA), 10mM dNTP mixture (Bioline, London, UK), and 0.5 µg of RNA at 65^{O}C for 5min and then adding 5X First-Strand Buffer, 0.1mM DTT, 40 units RNaseOUT Recombinant RNase Inhibitor (Invitrogen Corp., Carlsbad, CA) and 400 units SuperScript III Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). This mixture was incubated at 25^{O}C for 5 minutes and then at 50^{O}C for 60 minutes. The reaction was inactivated by heating at 70^{O}C for 15 minutes. Complementary DNA was stored at -20^{O}C until required for PCR. To exclude the presence of contaminating genomic DNA, all reverse transcription reactions included a control that lacked the Superscript III enzyme. Water was used as a negative control.

Quantitation of *CYP11A1* and *COX-2* mRNA levels were carried out using Real-Time PCR (RT-PCR). Oligonucleotide primers for *18S* (ribosomal housekeeping gene), *CYP11A1* and *COX-2* were designed using LightCycler Probe Design software from bovine-specific sequences obtained from a gene database (http://www.ncbi.nlm.nih.gov). Primers were obtained from Eurogentec Ltd. (Hampshire, UK) and are shown in table 1. Primers were first validated to ensure amplification of a single fragment of the expected size. This was done using fresh granulosa cell samples by semi-quantitative PCR as described below

Table 2.1. Primers used to amplify specific bovine transcripts

Gene Name	Gene Symbol	Primer	Sequence	Fragment size (bp)
18S	***18S***	Forward Reverse	5'-GGG-GAA-TCA-GGG-TTC-G-3' 5'-GCT-GGC-ACC-AGA-CTT-G-3'	209
P450scc	***CYP11A1***	Forward Reverse	5'-AGA-GAA-TCC-ACT-TTC-GCC-ACA-TC-3' 5'-GGT-CTT-TCT-TCC-AGG-TTC-CTG-AC-3'	237
COX2	***COX-2***	Forward Reverse	5'-TCC-TGA-AAC-CCA-CTC-CCA-A-3' 5'-TGG-GCA-GTC-ATC-AGG-CAC-AG-3'	242
VEGFR1	***VEGFR1***	Forward Reverse	5'- GGT CTT ACG GAG TGC T -3' 5'- AGC AGG TCA CCT AGT TT -3'	226
VEGFR2	***VEGFR2***	Forward Reverse	5'- AGA GAA GCC TTT CTG GCT GTC -3' 5'- CTG GTT CTG GCC CAAC -3'	379

Amplification of each bovine transcript was done using Quantace SensiMix dT kit (Quantace, London, UK). PCR reactions were performed in a total volume of 12.5µl, containing 1µl of cDNA and 11.5µl of SYBR® Green mastermix and specific primers (final concentration 200nM) using a quantitative PCR machine, Statagene Mx3000p. A set of standards were created using 3µl of cDNA from a pool of fresh granulosa cell samples and included the following dilutions: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128. Each sample was run in duplicate at a 1:20 dilution for quantification of *18S* and a 1:4 dilution for quantification of *CYP11A1* and *COX-2*. Also included in the PCR reaction were duplicate samples without cDNA template (non-template controls) and duplicate reverse transcriptase controls. PCR cycles consisted of 10 minutes at 95^{O}C, followed by 40 cycles of 15s at 95^{O}C, 60^{O}C for 30s and 72^{O}C for 30s.

RT-PCR results were analysed using Mx3000p software. Copy numbers from each sample were obtained from the corresponding Ct values (the first reaction cycle at which an increase in template amplification can be detected) by extrapolation from a Ct-log copy number plot obtained from the standard curve. *CYP11A1* and *COX-2*

values were normalised to the *18S* value for each sample. Within each experiment, *CYP11A1* and *COX-2* expression were calculated relative to the value in untreated controls which was taken as 1.

2.3.5 Semi-quantitative PCR of *VEGFR1* and *VEGFR2*

Semi-Quantitative PCR was done on three pools of fresh granulosa cells and one pool of follicle wall cells from follicles of various sizes (4 to 14mm). Semi Quantitative PCR conditions were: 4 min 30 sec at 95^{O}C, followed by 15 cycles (*18S*) or 30 cycles (*VEGFR1* and *VEGFR2*) of 30 sec at 95^{O}C, 30 sec at 55^{O}C and 30 sec at 72^{O}C. A final inactivation step at 72^{O}C for 4 minutes and 30 sec was also carried out. PCR products were run on a 2.5% agarose gel.

2.4 Statistical Analyses

For cell proliferation data, percentage increases over untreated controls were calculated in each experiment and used for statistical analyses. For any other endpoints, responses to treatments were expressed as fold-increase over values in untreated controls. Dixon's test was used to identify suspected outlier values within data sets. These values were then excluded from subsequent analyses. The Kolmogorov-Smirnov normality test was applied to each data set ($P < 0.01$). Data that were not normally distributed were log transformed. Data were then analysed by General Linear Model procedure using Minitab 15 statistical software package (Minitab Ltd. Coventry, UK) to determine main effect of treatment using each individual experiment as a block. If the main effect was significant ($P < 0.05$) or tended to be significant ($P < 0.1$), pair-wise multiple comparisons of the means were performed using Tukey's test to establish differences among groups. Comparisons involving only two treatment groups were done by unpaired T test. Significance was considered at $P < 0.05$ whereas values between $P = 0.1$ and $P = 0.05$ were considered as approaching significance.

2.5 Results

2.5.1 VEGFR expression in bovine granulosa cells

As detected by semi-quantitative PCR (figure 2.1), fresh bovine granulosa cell pools showed expression of both *VEGFR1* and *VEGFR2*. Transcript levels for the two receptors were lower in granulosa cells than in follicle wall lysates.

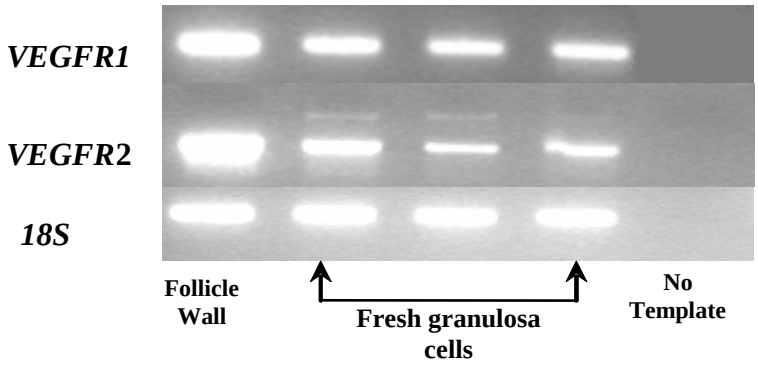


Figure 2.1. Specific PCR products for *VEGFR1, VEGFR2* and *18S* in pools of freshly collected walls and granulosa cells from bovine follicles 4 to 14 mm (n = 12 follicles).

2.5.2 Effects of VEGF on granulosa cell proliferation

A dose-dependent effect of VEGF treatment on granulosa cell proliferation was observed (Figure 2.2). On average, granulosa cell proliferation was significantly induced by 1 ng/ml VEGF dose (24% over untreated controls; $P = 0.024$) but there were no detectable responses to 10 or 100 ng/ml VEGF ($P > 0.1$). As expected, FSH (10ng/ml) induced a significant proliferative response (40%; $P < 0.0001$) whereas LH did not ($P > 0.1$). Co-administration of FSH (10ng/ml) and VEGF (1ng/ml) resulted in a cell proliferation response that was 2.4-fold that elicited by FSH alone ($P = 0.045$).

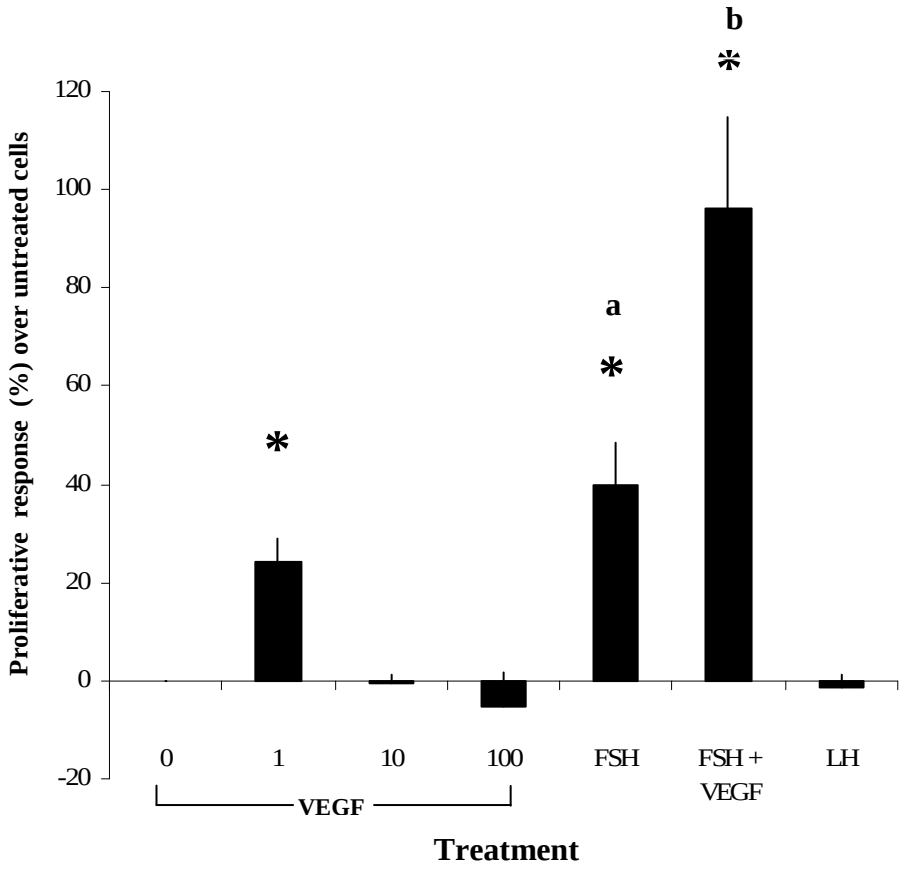


Figure 2.2. Proliferation response (mean % ± SEM; n = 3 to 12 experiments) of bovine granulosa cells collected from 4-8mm follicles to different VEGF doses (1, 10 and 100 ng/ml), FSH (10 ng/ml), a combination of FSH (10 ng/ml) and VEGF (1 ng/ml), and LH (10 ng/ml). There was an overall effect of treatment, $P < 0.0001$. A star denotes a significant difference from untreated control ($P < 0.05$). Means with different letters (a, b) are different ($P < 0.05$)

2.5.3 ERK1/2 phosphorylation in response to VEGF

Exposure of cultured bovine granulosa cells to 0, 1, 10 or 100 ng/ml VEGF resulted in a dose-dependent increase in p-ERK 1/2 ($p < 0.002$), with the highest response being induced by the 100 ng/ml dose (3.2-fold over untreated controls; Figure 2.3). On average, this response was about 65 % the response induced by 100 ng/ml EGF.

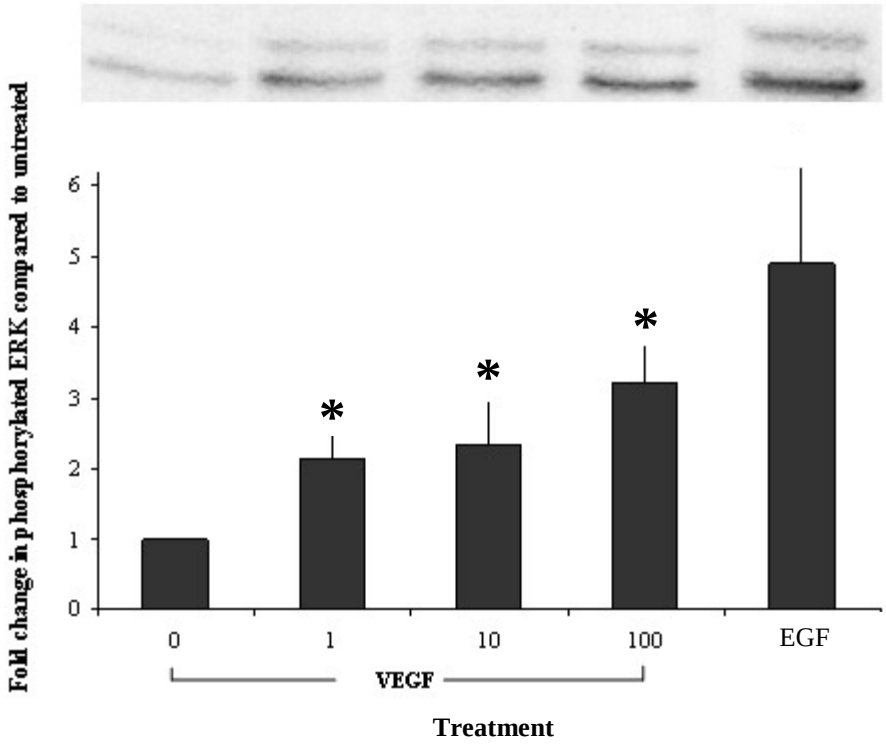


Figure 2.3 Representative immunoblot and mean (± S.E) band intensities of p-ERK 1/2 after treatment of bovine granulosa cells collected from follicles 4 to 8 mm with different doses of VEGF (1, 10 and 100 ng/ml; n = 3 to 9 experiments). EGF, 100 ng/ml treatment was included as a positive control. Statistical analyses was done of data from groups treated with VEGF (0 to 100 ng/ml) and indicated a significant effect of treatment ($P<0.002$). A star on a mean responses to VEGF treatment denotes a significant difference from untreated controls ($P < 0.03$).

2.5.4 Effects of VEGF on expression *of CYP11A1* and *COX-2*

Expression of *CYP11A1* in cultured bovine granulosa cells collected from follicles 4 to 8 mm did not change in response to VEGF concentrations of 1, 10 or 100 ng/ml ($P > 0.1$) (figure 2.4). In contrast, FSH (10 ng/ml; $P < 0.001$), but not LH ($P > 0.1$) significantly increased the expression of *CYP11A1*. The response to FSH (about 2.8-fold relative to controls) was not significantly affected by co-stimulation with VEGF (1 ng/ml), $P > 0.1$.

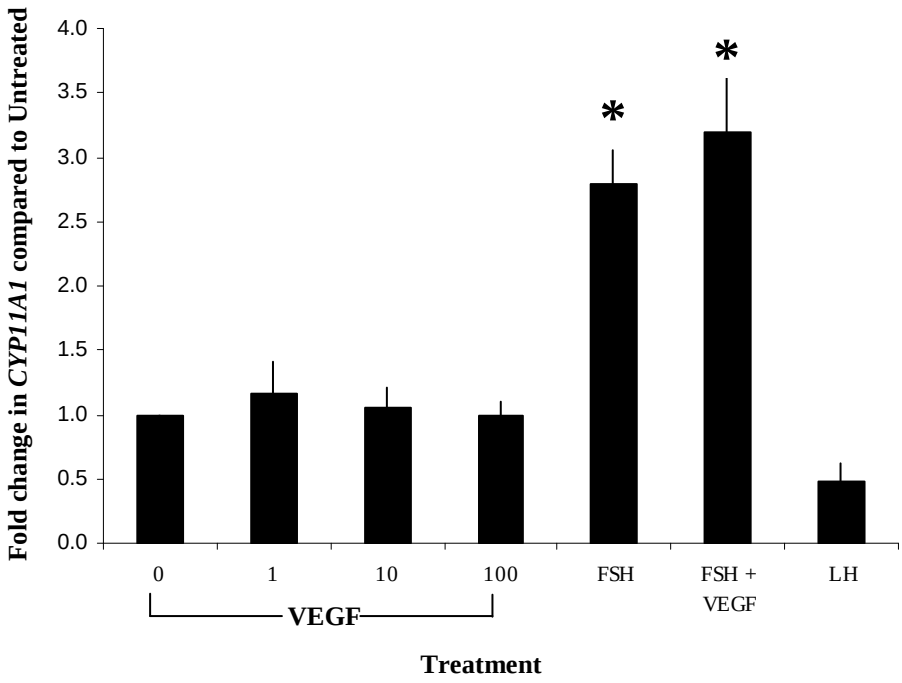


Figure 2.4. Fold-increase in *CYP11A1* expression (mean ± SEM; n = 4 to 13 experiments) in response to different doses of VEGF (1, 10 and 100 ng/ml), FSH (10 ng/ml), LH (10 ng/ml) or a combination of FSH (10 ng/ml) and VEGF (1 ng/ml) in cultured granulosa cells from bovine follicles 4 to 8 mm. There was an effect of treatment ($P < 0.00001$), A star denotes a significant difference from untreated controls ($P < 0.0001$).

Treatment of granulosa cells collected from 9 to 14 mm follicles with 1 ng/ml of VEGF induced a significant increase in *COX-2* expression (approximately 1.8 fold) compared to untreated control ($P = 0.003$) (figure 2.5). Neither of the 10 or 100 ng/ml VEGF doses had a significant effect on COX-2 expression ($P > 0.1$). The response to 1 ng/ml VEGF was similar to that induced by LH (100 ng/ml). However, co-stimulation of cells with LH (10 ng/ml) and VEGF (1 ng/ml) resulted in a significant reduction in COX-2 expression compared to LH (10 ng/ml) alone ($p < 0.0002$).

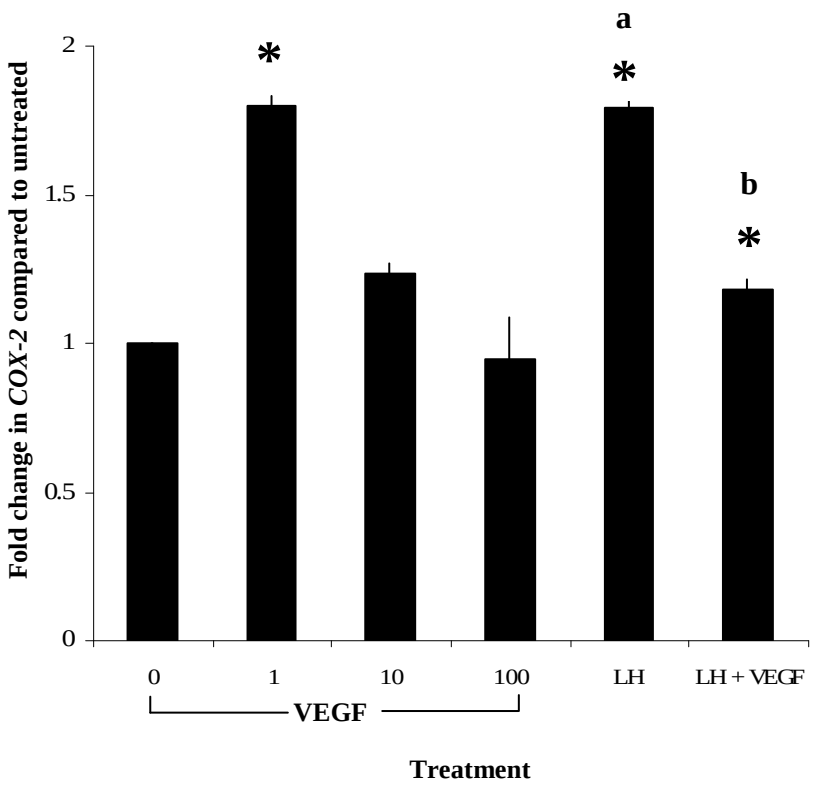


Figure 2.5. Fold-increase in *COX-2* expression (mean ± SEM; n = 3 to 8 experiments) in response to different doses of VEGF (1, 10 and 100 ng/ml), LH (10 ng/ml) or a combination of LH (10 ng/ml) and VEGF (1 ng/ml) in cultured bovine granulosa cells collected from 9-14mm follicles. There was a significant effect of treatment ($P < 0.0001$) A star denotes a significant difference from untreated control ($P < 0.004$). Means with different letters (a, b) are significantly different ($P < 0.002$).

2.5.5 Effect of ZM323881 on LH-induced ERK1/2 phosphorylation

Levels of p-ERK in granulosa cells from 9 to 14 mm follicles increased relative to controls in response to LH treatment ($P = 0.003$) but did not increase in LH-treated cells that were pre-incubated with the VEGFR2 inhibitor, ZM323881 (50μM; $P = 0.27$ Figure 2.6, **A)**. Exposure of granulosa cells to ZM323881 alone did not alter p-ERK levels ($P > 0.9$). As expected, an increase in p-ERK in response to VEGF occurred in the absence ($P = 0.0001$) but not in the presence ($P = 0.2694$) of ZM323881 (Figure 2.6, **B)**. In contrast, EGF stimulated p-ERK both in the absence ($P < 0.0001$) and presence ($P < 0.0001$) of ZM323881 (Figure 2.6, **C)**.

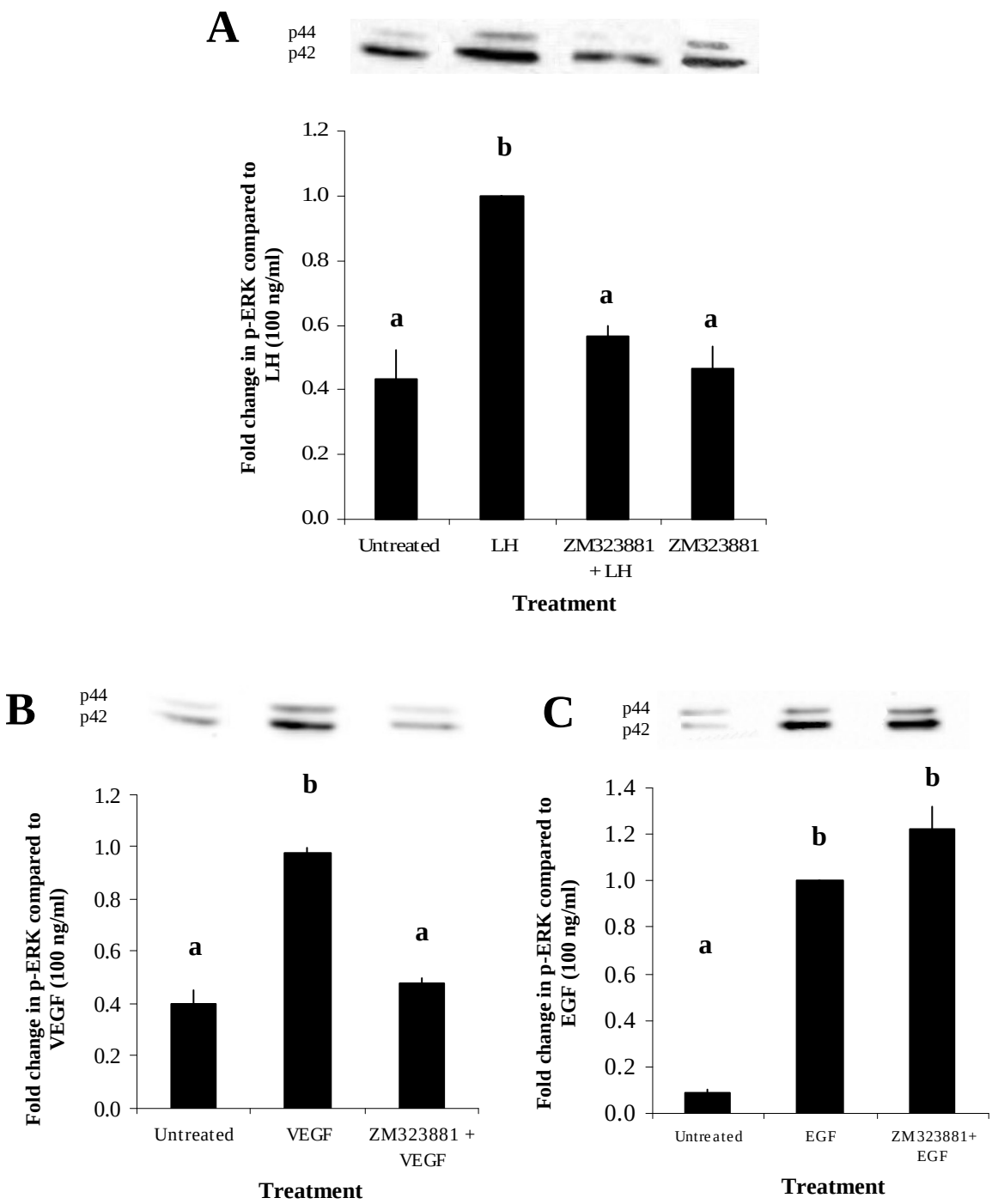


Figure 2.6. Representative Immunoblots and mean (± SEM, n = 3 to 5 assays) band intensities of p-ERK in cultured bovine granulosa cells collected from 9-14mm follicles and exposed to LH (100 ng/ml, **A**), VEGF (100 ng/ml, **B**) and EGF (100 ng/ml, **C)** in the presence or absence of ZM323881 (50μM). Within each graph, levels of p-ERK for each treatment group are shown relative to treatment with VEGF, LH or EGF. There was an overall effect of treatment on p-ERK levels in all cases (**A**: P = < 0.0001, **B**: P = 0.001, **C**: <0.0001). Means with different letters (a, b) within a graph are significantly different (P < 0.05). The same amount of protein was loaded for each sample.

2.5.6 Effect of ZM323881 on LH-induced *COX-2*

There was a significant increase in *COX-2* in response to LH stimulation of granulosa cells from 9 to 14mm follicles (100 ng/ml: P = 0.0007) that was abolished by pre-incubating cells with ZM323881 (50μM, P = 0.017) (figure 2.7). Treatment of granulosa cells with ZM323881 alone had no effect on *COX-2* (P > 0.9).

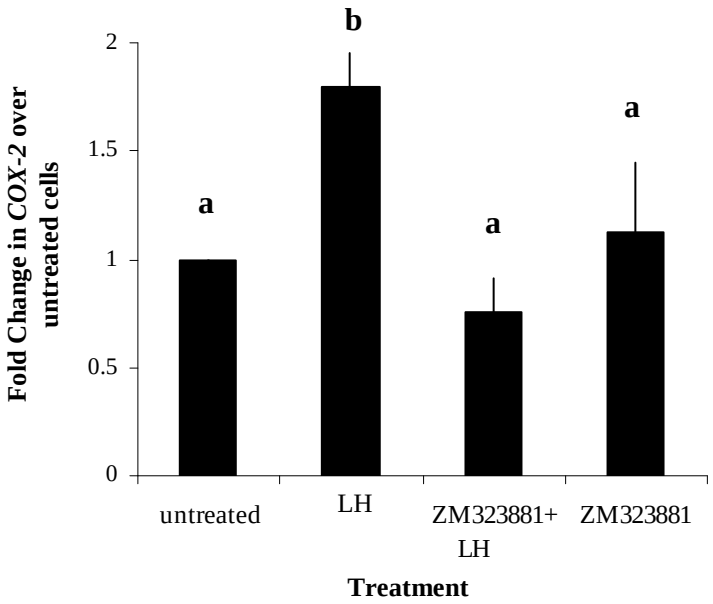


Figure 2.7. Fold increase in *COX-2* mRNA (mean ± SEM; n = 3 experiments) in cultured bovine granulosa cells collected from 9-14 mm follicles and exposed to LH (100 ng/ml) and/or ZM323881 (50μM). There was an overall effect of treatment (P = 0.001) on *COX-2* expression. Means with different letters (a, b) are significantly different (P < 0.05).

2.6 Discussion

Treatment of primates with VEGF antagonists has demonstrated that VEGF is an absolute requirement for the completion of follicle development (Hazzard, Xu, & Stouffer 2002) (Fraser, Wilson, Rudge et al. 2005). It has been observed that 1) the avascular granulosa cell layer is a major site of VEGF production within the follicle (Geva & Jaffe 2000; Grasselli, Basini, Bussolati et al. 2002), 2) these cells express VEGF receptors (Greenaway, Connor, Pedersen et al. 2004), an observation that we have confirmed in the present study and 3) the expression of both VEGF and VEGF receptors in granulosa cells markedly increase in selected (dominant) follicles (Ginther, Gastal, Gastal et al. 2004b; Greenaway, Connor, Pedersen et al. 2004). These observations led us to more precisely investigate in vitro the direct effects that physiological doses of VEGF, alone or in combination with gonadotrophins, may have in specific responses of granulosa cells during development and ovulation of the dominant follicle. Proliferative and steroidogenic responses to the follicle selection-associated increase in VEGF levels were investigated in vitro using granulosa cells from follicles 4 to 8 mm in diameter, corresponding to diameters just before selection of the dominant follicle, whereas potential effects of VEGF on the peri-ovulatory increase in COX2 expression were assessed in cells collected from follicles with a diameter close to that before ovulation (9 to 14 mm).

VEGF concentrations in bovine follicular fluid were reported to be about 1 ng/ml in follicles 5-7 mm and concentrations increased to 5 ng/ml in follicles 12-14 mm (Einspanier, Schonfelder, Muller et al. 2002). In another study (Grazul-Bilska, Navanukraw, Johnson et al. 2007) mean intrafollicular VEGF concentrations in oestrogen-active follicles with a mean diameter of 8.6 mm (corresponding to the beginning of dominance) were 1.2 ng/ml, whereas oestrogen-inactive follicles (mean diameter, 11.3 mm) had a mean VEGF concentration of 0.5 ng/ml. In comparison, VEGF levels in follicular fluid from preovulatory follicles have been reported to range between 0.7 and 7 ng/ml in primates (Asimakopoulos, Nikolettos, Papachristou et al. 2005; Anasti, Kalantaridou, Kimzey et al. 1998; Hazzard, Molskness, Chaffin

et al. 1999) and between 3 and 10 ng/ml in pigs (Barboni, Turriani, Galeati et al. 2000); (Galeati, Spinaci, Govoni et al. 2003). Thus, the doses of VEGF used in this study (1, 10 and 100 ng/ml) were chosen to encompass the expected range of concentrations found within the developing bovine dominant follicle.

To our knowledge, this study showed for the first time that VEGF dose-dependently induces proliferation of bovine granulosa cells. This is consistent with results with porcine granulosa cells (Grasselli, Basini, Bussolati et al. 2002) and with a variety of other non-endothelial cell types (Zhu, Jin, Mao et al 2003; Gaudio, Barbaro, Alvaro et al. 2006; Wilgus, Matthies, Radek et al. 2005; Chen, Qian, Wu et al. 2007). A concentration of VEGF of 1 ng/ml resulted in a significant proliferative response of granulosa cells whereas higher VEGF concentrations (10 and 100 ng/ml) abolished all proliferative effects. In a previous study, Greenaway et al (2004) found that VEGF increased the survival of granulosa cells but had no effect on cell proliferation. We speculate that the concentrations of VEGF used in the Greenaway study (50 and 100 ng/ml) were above the range of concentrations expected to stimulate cell proliferation.

In the present study, co-administration of FSH (10 ng/ml) and VEGF (1 ng/ml) resulted in a 2.4 fold increase in cell proliferation relative to FSH treatment alone, thus indicating that VEGF potentiates the effects of FSH. Consistent with this result, co-treatment of bovine cumulus-oocyte complexes with FSH and VEGF in vitro resulted in enhanced blastocyst formation compared to FSH alone (Einspanier, Schonfelder, Muller et al. 2002). The results on induction of cell proliferation by VEGF in the present study suggest that the differential increases in VEGF and VEGF receptor expression in granulosa cells during follicle selection (Einspanier, Schonfelder, Muller et al. 2002; Ginther, Gastal, Gastal et al. 2004b) act in combination with FSH to enhance cell proliferation thus promoting the continuous growth of the dominant follicle in a similar manner to what has been shown for other growth factors such as IGF-I (Gutierrez, Campbell, & Webb 1997b). Presumably, once VEGF concentrations (and VEGF receptor expression in granulosa cells) reach their highest levels in preovulatory follicles, a stimulatory effect on cell proliferation

may no longer occur, a suggestion that is in keeping with the cease in cell proliferation associated with the terminal differentiation of follicular cells during ovulation (Richards 1980).

Since VEGF stimulates proliferation of endothelial cells via VEGFR2 activation of ERK 1/2 (Byrne, Bouchier-Hayes, & Harmey 2005) we investigated whether VEGF induced ERK activation in granulosa cells. VEGF treatment did result in a dose-dependant increase in phosphorylation of ERK 1/2 with a maximal effect induced by the 100 ng/ml dose of VEGF. The apparent dissociation between ERK 1/2 activation and proliferative responses at the higher VEGF doses (10 and 100 ng/ml) may be explained by the role of ERK 1/2 signalling in both proliferation and differentiation responses of cells (Vaudry, Stork, Lazarovici et al. 2002), including granulosa cells in which an increase in ERK1/2 activity in preovulatory follicles mediates cell cycle arrest and terminal cell differentiation (Su, Nyegaard, Overgaard et al. 2006).

We then examined the potential involvement of VEGF in another granulosa cell response in selected follicles, P450scc expression. A predominant feature of the selection process is a pronounced increase in steroid production by the dominant follicle (Reviewed by Beg & Ginther 2006). In cattle, this increase is largely accounted for by an increase in mRNA for P450scc in granulosa and theca cells (Tian, Berndtson, & Fortune 1995). In the present study, in contrast to FSH, VEGF did not induce *CYP11A1* expression at any concentration used. In a study with porcine granulosa cells, however, VEGF induced an increase in oestradiol production by granulosa cells (Grasselli, Basini, Bussolati et al. 2002). Whether induction of oestradiol production by VEGF involved an increase in P450scc was not analysed in that study. The precise role of VEGF in follicular cell steroidogenesis and whether such a role varies in different species warrants further investigation.

We also examined the effects of VEGF on a hallmark granulosa cell response associated with ovulation, COX2 expression. COX2 acts as a key rate-limiting step in the production of prostaglandins which are critical for the rupture of the preovulatory follicle in response to the ovulatory LH surge (Reviewed by Espey &

Richards 2006). COX2 is a component of the temporal inflammatory response associated with ovulation and as such is only transiently induced in the ovulatory follicle in response to the ovulatory gonadotrophin surge, and this occurs both in vivo and in vitro (Hedin, Gaddy-Kurten, Kurten et al. 1987; Duffy & Stouffer 2001; Joyce, Pendola, O'Brien et al. 2001; Richards 1997; Sirois 1994; Sirois & Dore 1997; Sirois & Richards 1992; Wong & Richards 1991). In bovine ovulatory follicles the induction of COX2 occurs some 28-30 hours post-hCG treatment (Sirois, Sayasith, Brown et al. 2004). VEGF has been shown to be involved in upregulation of COX2 in endothelial (Tamura, Sebastian, Gurates et al. 2002) and non-endothelial cells (Wijayagunawardane, Kodithuwakku, Yamamoto et al. 2005). Treatment of bovine oviductal cells, with 1 ng/ml VEGF induced a significant increase in COX2 as well as stimulating the synthesis and release of prostaglandins E2 and F2α and Endothelin-1, leading to the conclusion that VEGF may enable proper oviduct contraction and transport of gametes in the oviduct (Wijayagunawardane, Kodithuwakku, Yamamoto et al. 2005). In the present study we found that treatment of cultured granulosa cells from bovine pre-ovulatory size follicles (9-14 mm in diameter) with 1 ng/ml VEGF stimulated a significant increase in COX2 (about 1.8-fold relative to controls). This increase was similar to that induced by treatment with LH (10 ng/ml), suggesting that VEGF, in addition to LH, may regulate the peri-ovulatory increase in follicular COX2. The reason for the absence of a COX2 response to stimulation of granulosa cells simultaneously with VEGF and LH in the present study is not known, although it is conceivable that a stimulation threshold exists above which VEGF is no longer induced. This possibility would be consistent with the need for fine-tuned intracellular mechanisms that ensure that the peri-ovulatory increase in COX2 expression is only transient.

The results on COX2 induction in the present study raised the possibility of a potential interplay between VEGF and LH in preovulatory follicles similar to that described between LH and the EGF system (Conti, Hsieh, Park et al. 2006). In particular, the EGF receptor has been shown to mediate the LH induction of specific ovulatory-associated responses, among them COX2 induction. To address this, we evaluated the effects of specific VEGFR2 inhibition with ZM323881 on LH

responses in granulosa cells from pre-ovulatory size follicles (9 to 14mm), namely phosphorylation of ERK1/2 and induction of COX2. The induction of p-ERK1/2 by 100 ng/ml LH was consistent with previous results on granulosa cells from preovulatory follicles (Salvador, Maizels, Hales et al. 2002) and with the reported role of ERK1/2 activation in mediating LH-induced differentiation of granulosa cells (Su, Nyegaard, Overgaard et al. 2006). Inhibition of VEGFR2 severely restricted the ability of LH to stimulate ERK phosphorylation in granulosa cells (approximately 1.67-fold) compared to LH alone (2.5-fold). The observed effect was presumably specific to VEGFR2 because the same inhibitor also abolished ERK1/2 activation by VEGF but not by a different RTK, (EGFR). Previous studies showed that inhibition of the EGFR has similar effects on gonadotrophin-induced ERK1/2 activation in granulosa cells (Andric & Ascoli 2006). The effects of VEGFR inhibition on immediate cell signalling responses (p-ERK1/2) to LH was associated with similar effects on a longer-term response, namely COX2 induction, as inhibition of VEGFR2 completely abolished the ability of LH to stimulate COX2 mRNA. These findings are consistent with an involvement of ERK1/2 activation on COX2 induction in cumulus granulosa cells (Hsieh & Conti 2005).

The mechanism(s) by which VEGFR may mediate the effects of LH in these cells needs to be examined. In relation to the EGFR, a model has been proposed whereby LH stimulation of granulosa cells in the pre-ovulatory follicle induces the expression of EGF-like growth factors which then act in a paracrine or juxtacrine manner to activate the EGFR (Conti, Hsieh, Park et al. 2006). It has been shown that for LH to elicit maturation of the mouse oocyte EGFR must be phosphorylated (Park, Su, Ariga et al. 2004). An additional mechanism by which EGF seems to mediate some of the actions of LH in follicles is LH-induced shedding of cell membrane-bound EGF-like growth factors that could then act on target cells (Andric & Ascoli 2006). Such release or ‘shedding’ of growth factors from the extracellular matrix (ECM) is believed to involve specific membrane-bound matrix metalloproteinases that induce proteolytic cleavage of growth factor molecules at the cell surface (Ashkenazi, Cao, Motola et al. 2005; Hsieh & Conti 2005; Prenzel, Zwick, Daub et al. 1999; Roelle, Grosse, Aigner et al. 2003).

Based on previous results, any or both of the two mechanisms outlined above, i.e., induction of growth factor expression in follicular cells and stimulation of growth factor release from follicular cell membrane stores, may explain the effects of VEGFR on LH-induced COX2 observed in this study. In particular, LH is known to stimulate VEGF synthesis from granulosa cells both in vivo and in vitro (Christenson & Stouffer 1997; Schams, Kosmann, Berisha et al. 2001). In addition, as in the case of EGF-like growth factors, large molecular weight isoforms of VEGF are bound to the ECM whereas small (bioactive) isoform are freely diffusible (Park, Keller, & Ferrara 1993). In this way, the ECM can sequester and act as a reservoir for VEGF (Houck, Leung, Rowland et al. 1992). Cleavage of VEGF from the ECM has been reported to involve the action of matrix metalloproteinases (Bergers, Brekken, McMahon et al. 2000; Lee, Jilani, Nikolova et al. 2005; Rodriguez-Manzaneque, Lane, Ortega et al. 2001). Although the specific MMPs involved in ligand-shedding depends on the cell type (Hsieh & Conti 2005), one interesting observation is that MMP9 seems to be commonly involved in cleavage of both EGF and VEGF (Bergers, Brekken, McMahon et al. 2000; Lee, Jilani, Nikolova 2005; Rodriguez-Manzaneque, Lane et al. 2001; Roelle, Grosse, Aigner et al. 2003). It would therefore be of interest to further investigate the involvement of MMP9 in VEGFR transactivation using specific MMP inhibitors. In other studies, matrix metalloproteinase activity has been shown to be required for LH-mediated EGFR stimulation (Ashkenazi, Cao, Motola et al 2005; Panigone, Hsieh, Fu et al. 2008; Woods & Johnson 2007) where inhibition of MMP activity abolished or severely reduced the LH-induced phosphorylation of EGFR. Therefore, based on the findings above, it can be hypothesized that the LH preovulatory surge acts to increase VEGF expression by granulosa cells and at the same time stimulates the release of pre-existing VEGF from ECM stores. This increases the availability of bioactive VEGF to induce some of the responses associated with ovulation. Further studies will be needed to test this hypothesis.

In contrast to the effects on COX2 expression, the effects of VEGFR inhibition on LH-induced p-ERK1/2 in granulosa cells were exerted within a very short period of

time (<20 mins) and therefore may not be explained by stimulation of *de novo*-production of VEGF by LH. In a previous study, an MMP inhibitor abolished an immediate increase (within 20 min) in p-ERK1/2 in response to LH (Andric & Ascoli 2006) suggesting that release of growth factor ligand from ECM stores is involved in this response. An additional mechanism that may explain the role of VEGFR on the immediate activation of p-ERK1/2 by LH involves Src tyrosine kinases. Numerous examples have been described of Src tyrosine kinases playing a role in transactivation of RTKs by GPCRs (Luttrell & Luttrell 2004), and it is known that Src TKs can be activated by gonadotrophins in granulosa cells (Wayne, Fan, Cheng et al. 2007). The involvement of Src tyrosine kinases in transactivation of RTKs by the LH surge in granulosa cells should be explored further.

In summary, a concentration of VEGF (1 ng/ml) equivalent to levels found physiologically in bovine dominant follicles acted to induce proliferation of bovine granulosa cells from 4 to 8 mm follicles and did not increase the expression of the steroidogenic enzyme, P450scc. The effect of VEGF on proliferation was associated with an increase in ERK1/2 activation in those cells. Treatment of granulosa cells from dominant-size follicles (9 to 14 mm in diameter) with the same dose of VEGF up-regulated the expression of COX2. In addition, pre-treatment of these cells with a selective VEGFR2 inhibitor (ZM323881) resulted in a significant reduction in the ability of LH to induce ERK phosphorylation and a complete block on the ability of LH to stimulate COX2 expression. The results of this study clearly demonstrate potentially important roles of VEGF, independent of its angiogenic effects, at different stages during the development of the dominant follicle, and particularly suggest a role of VEGFR2 in mediating ovulatory responses induced by the gonadotrophin surge similar to what has been described for EGFR.

CHAPTER 3:

Expression of Gα subunits and PLCβ isoforms in bovine granulosa cells throughout antral follicle development.

3.1 Abstract

Previous reports suggest that VEGF exerts its effects through not only Receptor Tyrosine Kinases (RTKs) but also via interactions with specific heterotrimeric G-proteins and PLCβ isoforms. To investigate whether such intracellular cross-talk may mediate some of the effects of VEGF shown in Chapter 2, we first examined the expression of heterotrimeric G-protein α subunits *(GNAS, GNAQ, GNA11,* and *GNAI1, 2* and *3)* and PLCβ isoforms 1 to 4 in bovine granulosa cells throughout follicle development. Results showed that *GNAS*, *GNA11* and *GNAI2* were all expressed at significantly ($P < 0.05$) higher levels in granulosa cells of large follicles (10.0 to 13.9 mm) than in cells from small (2.0 to 5.9 mm) and medium (6.0 to 9.9 mm) size follicles. In addition, all PLCB isoforms except *PLCB2* were expressed in bovine granulosa cells with PLCB3 being more abundant than *PLCB 1* and *4*. Levels of *PLCB3* in granulosa cells from large follicles were very high (>16-fold; $P < 0.005$) compared to medium or small follicles. Immunocytochemical analysis revealed that *PLCB3* was located primarily in the cytoplasm, whereas PLCB1 was distributed primarily in the nucleus. In conclusion, this study identified *GNAS*, *GNA11* and *GNAI2* and *PLCB3* as potentially involved in cross-talk between VEGF and G protein signalling pathways during the development of the dominant follicle.

3.2 Introduction

The studies described in Chapter 2 indicated that, in addition to directly stimulating bovine granulosa cell responses, VEGF modulates the effects of gonadotrophins on these cells. Specifically, when granulosa cells were co-stimulated with VEGF and gonadotrophins, VEGF enhanced the proliferative effects of FSH and inhibited LH-induced expression of COX2. These results prompted the investigation of potential cross-talking between VEGF and gonadotrophin signalling pathways in these cells, as described in the present chapter and Chapter 4.

The receptors for FSH (FSHR) and LH (LHR) belong to the G-protein-coupled receptor (GPCR) superfamily and signal primarily through adenylate cyclase activation by the heterotrimeric G protein type, Gs, and possibly through an additional pathway involving Gi/Gq activation of Phospholipase Cβ (PLCβ) (Herrlich, Kuhn, Grosse et al. 1996; Kuhn & Gudermann 1999; Rajagopalan-Gupta, Lamm, Mukherjee et al. 1998). VEGF is recognised to elicit its effects via two receptor tyrosine kinases (VEGFR1 and VEGFR2), which signal through activation of Phospholipase Cγ (PLCγ) and Raf-MEK-MAP pathways (Petrova, Makinen, & Alitalo 1999). Cross-talk between GPCR and RTK signalling pathways is well characterised in relation to GPCR transactivation of RTKs (Reviewed by Luttrell, Daaka, & Lefkowitz 1999). This includes LH transactivation of EGF receptor, a phenomenon that has been shown to be involved in granulosa differentiation and oocyte activation during the peri-ovulatory period (Reviewed by Conti, Hsieh, Park, & Su 2006).

Accumulating evidence indicates that cross-talk between GPCR and RTKs may also occur in the other direction, that is, through activation of specific hetrotrimeric G proteins by different RTKs (Reviewed in Patel 2004)). In the case of VEGFR, Mukhopadhyay and associates provided evidence that in endothelial cell lines, VEGF induced cell proliferation and migration via VEGFR2 interactions with Gαq/11 and PLC-β (rather than via the classical RTK-activated PLC-γ pathway) (Mukhopadhyay, Zeng, & Bhattacharya 2004). In the same study, it was shown that

the Gi inhibitor, pertussis toxin (PTX), abolished VEGFR1 mediated MAPK phosphorylation but had no effect on VEGFR2 mediated MAPK phosphorylation. Those results indicated that Gq/11 proteins are involved in VEGFR2-mediated responses, whilst PTX-sensitive Gi/o proteins are involved in responses mediated by VEGFR1. In another study, a requirement for another heterotrimeric G-protein, Gα13, during VEGF stimulated endothelial cell migration was reported (Shan, Chen, Wang, Tan, Gu, & Huang 2006). Overall, these observations are important because they indicate signalling roles for heterotrimeric G proteins in addition to their known involvement in GPCR pathways. Whether VEGFR activation of heterotrimeric G-proteins is a more general signalling mechanism involved in the physiological effects of VEGF in other cell types has not been reported to date. In that regard, granulosa cells provide a suitable primary culture model to study such interactions.

Human granulosa cells (Lopez Bernal, Bellinger, Marshall et al. 1995; Saltarelli 1999) and porcine follicular membranes (Rajagopalan-Gupta, Lamm, Mukherjee et al. 1998) were shown to express the G protein types, Gαs, Gαi1, Gαi2, Gαi3, Gαq and Gα11, whereas in addition, bovine luteal membranes were found to express, Gα12 and Gα13 (Herrlich, Kuhn, Grosse et al. 1996). Expression of Gαo was found in some of these studies (Saltarelli 1999) but not in others (Herrlich, Kuhn, Grosse et al. 1996; Lopez Bernal, Bellinger, Marshall et al. 1995). All known isoforms of PLCβ (PLCβ1 through 4), which are activated by G proteins, were reportedly identified in porcine granulosa cells (Lieberherr, Grosse, & Machelon 1999).

Any of these G protein types and PLCβ isoforms may be involved in mediating the actions of VEGF on granulosa cells during the development of the dominant follicle. To address this question more precisely, temporal expression patterns of Gα subunits and PLCβ isoforms in bovine granulosa cells during follicle development need to be characterised and to our knowledge this has not been done. The aims of this study were therefore to 1) establish which G protein α subunits and PLCβ isoforms are expressed by bovine granulosa cells and 2) determine changes in expression of these signalling molecules throughout the development of the dominant follicle.

3.3 Materials and Methods

3.3.1 Collection of fresh granulosa cell samples for gene expression

Granulosa cells were obtained from bovine ovaries as described in Chapter 2 and kept on ice throughout to prevent protein and RNA degradation. Granulosa cells were isolated from follicles of different size categories as small (2.0 to 5.9 mm), medium (6.0 to 9.9 mm) and large (10.0 to 13.9 mm). The largest follicle of a wave becomes dominant when it reaches 8.0 to 9.0 mm (Ginther 2000), therefore, the chosen diameter ranges corresponded to before the emergence of the dominant follicle (small follicles), beginning of dominance (medium follicles), and established dominance or preovulatory stage (large follicles). Fourteen different sets of samples including the three diameter categories were obtained from different ovarian collections on different days. Within each set, separate granulosa cell pools were obtained from small follicles (5 to 27 follicles per pool) and medium follicles (4 to 20 follicles per pool), whereas large follicles were processed individually. This was done to increase the number of cells collected from small and medium follicles to numbers comparable to those from large follicles. Half of the cells obtained from each diameter range were processed for RNA extraction and the other half were used for isolation of protein.

3.3.2 QPCR analysis of Gα subunits and PLCB isoforms

RNA extraction, cDNA synthesis, primer validation and QPCR were carried out according to the methodologies described in chapter 2. Primers (Table 3.1) were obtained from Eurogentec Ltd. (Hampshire, UK). Messenger RNA levels for Gαs, Gαq, Gα11, Gαi1, Gαi2 and Gαi3 were analysed because these specific Gα subunits have been previously identified in follicular cells from human and pig (Lopez Bernal, Bellinger, Marshall, Phaneuf, Europe-Finner, Asboth, & Barlow 1995) (Rajagopalan-Gupta, Lamm, Mukherjee, Rasenick, & Hunzicker-Dunn 1998). The four PLCβ isoforms were also analysed. *LHR* was quantified because is a hallmark of healthy, growing dominant follicles (Bao & Garverick 1998;Bao, Garverick, Smith et al. 1997). Data from large follicles that had *LHR* expression levels less than 3-fold

higher the levels in medium follicles from the same collection were discarded. This criterion was based on results from previous studies on *LHR* expression during follicular waves (Beg & Ginther 2006).

Table 3.1. Primers used for qPCR of specific transcripts in bovine granulosa cells.

Gene Name	Gene symbol	Primer	Sequence	Fragment size (bp)
18S	*18S*	Forward	5'-GGG-GAA-TCA-GGG-TTC-G-3'	
		Reverse	5'-GCT-GGC-ACC-AGA-CTT-G-3'	209
LHR	*LHR*	Forward	5'-AC-ACA-TAA-CCA-CCA-TAC-GAC-3'	
		Reverse	5'-GGA-CTC-TAG-CCC-GTA-GG-3'	256
Gαs	*GNAS*	Forward	5'-CTC-GCT-ACA-CTA-CTC-CTG-3'	
		Reverse	5'-CAG-CTC-ATA-CTG-ACG-G-3'	233
Gαq	*GNAQ*	Forward	5'-CCA-CCT-AGT-TGA-CTA-TTT-CCC-3'	
		Reverse	5'-CGA-ACA-CAA-AGC-GGA-T-3'	167
Gα11	*GNA11*	Forward	5'-GCC-CTT-AGT-GAG-TAC-GAC-3'	
		Reverse	5'-CGA-ACT-CCG-GGA-AGT-AG-3'	196
Gαi1	*GNAI1*	Forward	5'-GAT-CCC-GAG-AGT-ACC-AG-3'	
		Reverse	5'-AGA-ACC-AGG-TCA-TAG-TCA-C-3'	277
Gαi2	*GNAI2*	Forward	5'-GCG-ACT-ACA-TCC-CTA-CG-3'	
		Reverse	5'-TGT-TGT-TGC-AGA-TAC-TGT-CA-3'	276
Gαi3	*GNAI3*	Forward	5'-ATT-AAA-CGG-TTA-TGG-CGA-G-3'	
		Reverse	5'-CGT-CCG-CAA-AAC-GTC-T-3'	153
PLCβ1	*PLCB1*	Forward	5'-ACA-ACG-TCC-CTG-ACA-C-3'	
		Reverse	5'-CAA-TCG-TCT-GCG-CTT-C-3'	133
PLCβ2	*PLCB2*	Forward	5'-CTG-TCT-GCA-CAA-CGA-G-3'	
		Reverse	5'-CCC-GTT-GCT-TGT-TGG-A-3'	256
PLCβ3	*PLCB3*	Forward	5'-CGA-GAA-GTA-CGA-ACC-C-3'	
		Reverse	5'-GAG-ATA-GGT-GTT-GTG-CGA-3'	181
PLCβ4	*PLCB4*	Forward	5'-TGT-GGG-TGC-TAC-CAC-T-3'	
		Reverse	5'-CCT-CCC-TTG-GGG-TAA-AT-3'	219

Within each sample set, before statistical analyses expression data for each follicle size were normalised to the corresponding value for small follicles which was taken as 1.

3.3.3 Western Blotting Analysis of Gα subunits and PLBβ3

Protein lysates were processed and western blotting procedure was carried out using the methodologies previously described in chapter 2 with the following modifications. Cell lysis was carried out in the absence of phosphatase inhibitors. Protein lysates were run on separate 7.5% and 12% SDS-Polyacrylamide gels for subsequent immunoblotting with PLCβ3 and Gα antibodies, respectively (table 3.2). Following blocking, membranes were incubated overnight at room temperature with the appropriate antibody (see table 2). Prior to this incubation, membranes derived from 7.5% gels were cut horizontally into two portions for separate detection of PLCB3 (150kDa) and β-Tubulin (55kDa). Following antibody incubations, membranes were washed, imaged and analysed as described in chapter 2. Membranes derived from 12% gels were stripped and re-blotted to allow sequential detection of Gαs, Gαq/11, Gαi and β-Tubulin. Stripping was done by rinsing membranes twice for 5 minutes in TBS and then washing for 2 hours with constant agitation in stripping solution (0.2M Glycine pH 2.0, 0.1% SDS, 0.1% Tween-20). Membranes were then washed twice in TBS for 10 minutes before they were incubated in 3% blocking buffer for 2 hours and subsequently re-probed with the appropriate primary antibody.

3.3.4 Immunocytochemical Analysis of PLCB1 and 3.

Immunocytochemistry was performed to investigate the sub-cellular localisation of PLCβ1 and 3. Bovine granulosa cells from large follicles were plated in glass Lab-Tek chamber slides (Nunc Inc., Rochester, NY, USA) that had been pre-coated with fibronectin (Sigma) at a density of 0.2 million cells per chamber. Following 24 hours in culture, cells were washed with Phosphate Buffered Saline (PBS) and fixed with 500µl 4% Paraformaldehyde (PFA) for 20 minutes at room temperature. Cells were again washed with PBS and permeabilised with 500µl 100% ethanol for 5 minutes at room temperature. Cells were washed twice with PBS. Five hundred µl blocking buffer (10% Normal Goat Serum (NGS), 0.1% Tween in PBS) were then added to

each chamber and incubated at room temperature for 1 hour. After aspiration of blocking buffer, anti-PLCβ1 (1:50 diluted in blocking buffer solution, anti-rabbit, Santa Cruz) or anti-PLCβ3 (1:200 diluted in blocking buffer solution, anti-rabbit, Santa Cruz) antibody was added to each chamber (table3.2). Chamber slides were incubated at $4^{O}C$ overnight with gentle agitation. The following day, cells were washed twice with PBS for 5 minutes. One hundred µl of secondary antibody was added (1:80 diluted in PBS, Anti-Rabbit IgG Fluorescein Isothiocyanate (FITC) conjugate, Sigma) to each chamber and incubated in the dark at room temperature for 1 hour. In each slide, one control chamber was included containing PBS instead of primary antibody. Following incubation, cells were washed twice with PBS for 10 minutes. Chamber walls were removed and 25µl of VECTASHIELD with DAPI, 4', 6 – diamidino-2-phenylindole (Vector Laboratories Inc, Bulingame, CA, USA) were dispensed onto each slide and a cover slide was applied prior to viewing using a Nikon EC1 inverted microscope (Nikon, Melville, N.Y, USA). Images were processed using Nikon EZ-C1 software.

Table 3.2. Details of antibodies used in western blotting and immunocytochemistry experiments

Target	MW (kDa)	Antibody dilution	Source	Supplier
β-Tubulin	55	1:1000	Rabbit	Cell Signalling Technology Inc
Gαs	43-45 & 48-52	1:1000	Rabbit	Millipore/Upstate
Gαq/11	42	1:500	Rabbit	Santa Cruz Biotechnology
Gαi 1/2	40	1:1000	Rabbit	Millipore/Upstate
PLCβ1	150	1:50	Rabbit	Santa Cruz Biotechnology
PLCβ3	150	1:200	Rabbit	Santa Cruz Biotechnology

3.4 Statistical Analysis

Dixon's test was used to identify suspected outlier values within data sets ($P < 0.01$). These values were then excluded from subsequent analyses. The Kolmogorov-Smirnov normality test was applied to each data set to asses whether data were normally distributed. Data not normally distributed ($P < 0.01$) were log transformed. Data were then analysed by General Linear Model using Minitab 15 statistical software package (Minitab Ltd. Coventry, UK) to determine the main effect of

follicle size in each case and using sample set as a block. If follicle size effect was significant ($P < 0.05$) or tended to be significant ($P < 0.1$), pairwise comparisons of the means were performed using Tukey's test ($P < 0.05$).

3.5 Results

3.5.1 Gα subunit and LH Receptor mRNA levels during follicle development

3.5.1.1 *LHR and GNAS*

A total of 9 large follicle samples were discarded because of their reduced *LHR* expression, according to criteria indicated above. In the remaining samples, an overall mean increase in *LHR* expression in fresh bovine granulosa cells during follicle development was observed ($P < 0.001$; Figure 3.1**A**). Granulosa cells from small follicles expressed only residual levels of *LHR*, while granulosa cells from large follicles expressed *LHR* at levels that were approximately 250 fold greater than found in granulosa cells of medium follicles ($P < 0.001$). There was an overall effect of follicle size on *GNAS* expression in granulosa cells ($P = 0.04$; Figure 3.1B) with approximately 1.4-fold higher *GNAS* expression in large than in small or medium follicles ($P < 0.05$).

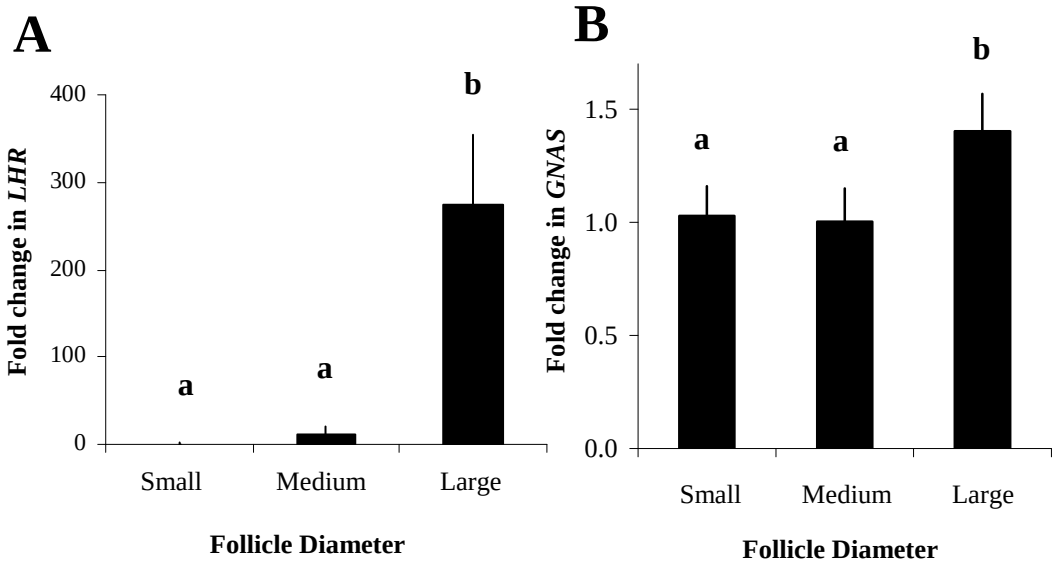


Figure 3.1. *LHR* (**A**) and *GNAS* (**B**) levels (mean ± SEM) in fresh bovine granulosa cells from small, medium and large follicles (n = 5 to 13 pools per diameter category). Within each sample set, expression levels for each diameter category were calculated relative to the value in small follicles, which was taken as 1. There was an effect of follicle size on *LHR* ($P < 0.001$) and *GNAS* (**P** = 0.04). Within a graph, means with different letters (a, b) are significantly different ($P < 0.05$).

3.5.1.2 *GNAQ* and *GNA11*

There was no overall effect of follicle size on *GNAQ* expression (P = 0.2; Fig 3.2**A**), although mean levels were approximately 1.6 fold higher in granulosa cells from large compared with small follicles. There was an effect for *GNA11* (P = 0.027; Fig 3.2**B**) with approximately 1.5–fold higher levels of *GNA11* in granulosa cells of large than small follicles (P < 0.05).

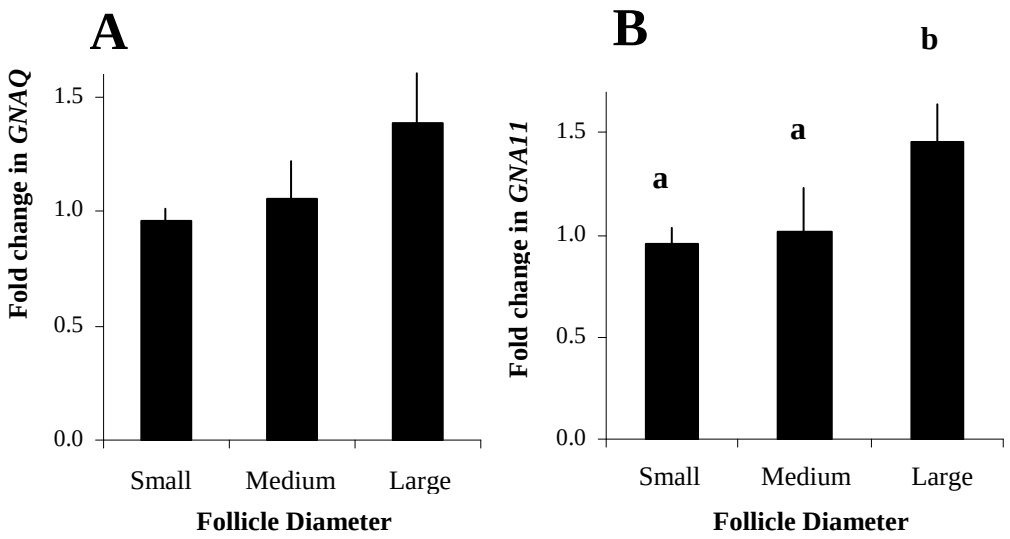


Figure 3.2. *GNAQ* (**A**) and *GNA11* (**B**) levels (mean ±) in fresh bovine granulosa cells from small, medium and large follicles (n = 5 to 13 pools per diameter category). Within each sample set, expression levels for each diameter category were calculated relative to the value in small follicles, which was taken as 1. Levels of each gene are shown relative to the value in small follicles. There was an overall effect on *GNA11* (P = 0.027). Within each graph, means with different letters (a, b) are significantly different (P < 0.05).

3.5.1.3 *GNAI 1, 2* and *3*

There was no effect (P > 0.5) of follicle size on *GNAI1* (Figure 3.3 **A**) or *GNAI3* (Figure 3.3 **C**) expression in granulosa cells. There was however an overall effect of follicle size on *GNAI2* expression (P = 0.03; Figure 3.3 **B**,) with approximately 1.6-fold higher levels of *GNAI2* expression in granulosa cells from large than small or medium follicles (P < 0.05).

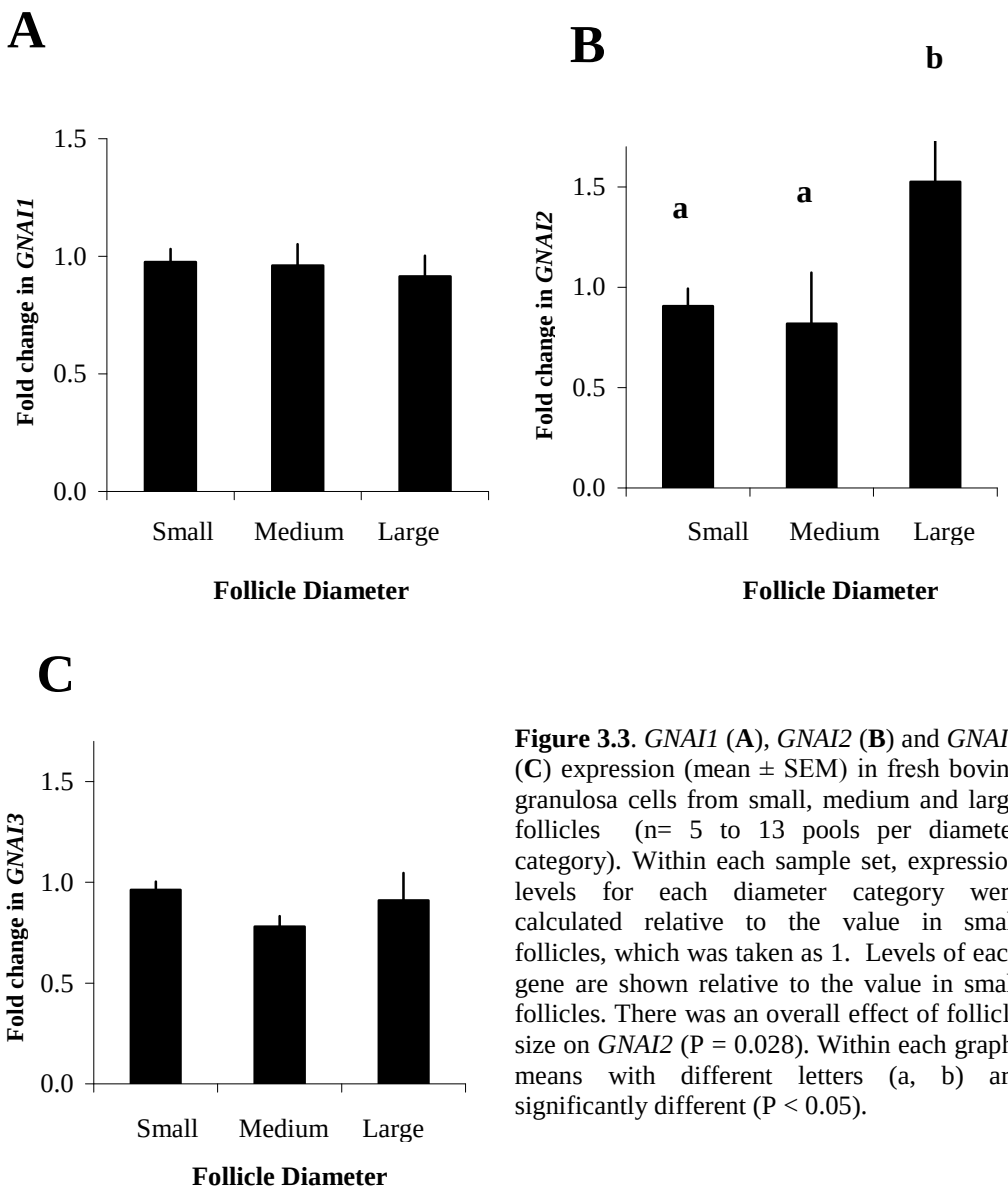


Figure 3.3. *GNAI1* (**A**), *GNAI2* (**B**) and *GNAI3* (**C**) expression (mean ± SEM) in fresh bovine granulosa cells from small, medium and large follicles (n= 5 to 13 pools per diameter category). Within each sample set, expression levels for each diameter category were calculated relative to the value in small follicles, which was taken as 1. Levels of each gene are shown relative to the value in small follicles. There was an overall effect of follicle size on *GNAI2* (P = 0.028). Within each graph, means with different letters (a, b) are significantly different (P < 0.05).

3.5.2 Gα subunit protein levels during follicle development

Representative blots for GNAS (short form and long forms, Figure 3.4**A**), GNAQ/GNA11 (Figure 3.4**B**) and GNAI1/GNAI2 (Figure 3.4**C**) are shown together with the corresponding β-Tubulin bands (loading control).

There was an overall effect of follicle size for both GNAS ($P < 0.0001$) and GNAQ/GNA11 ($P = 0.03$) levels but not for GNAI1/GNAI2 levels ($P = 0.28$). Granulosa cells from large follicles contained approximately 2-fold higher GNAS than cells from small or medium follicles ($P < 0.05$). Similarly, GNAQ/GNA11 levels were approximately 2-fold higher for large than for small or medium follicles ($P = 0.003$), whereas GNAI1/GNAI2 levels were 1.6 fold higher (non-significant) in large than small follicles.

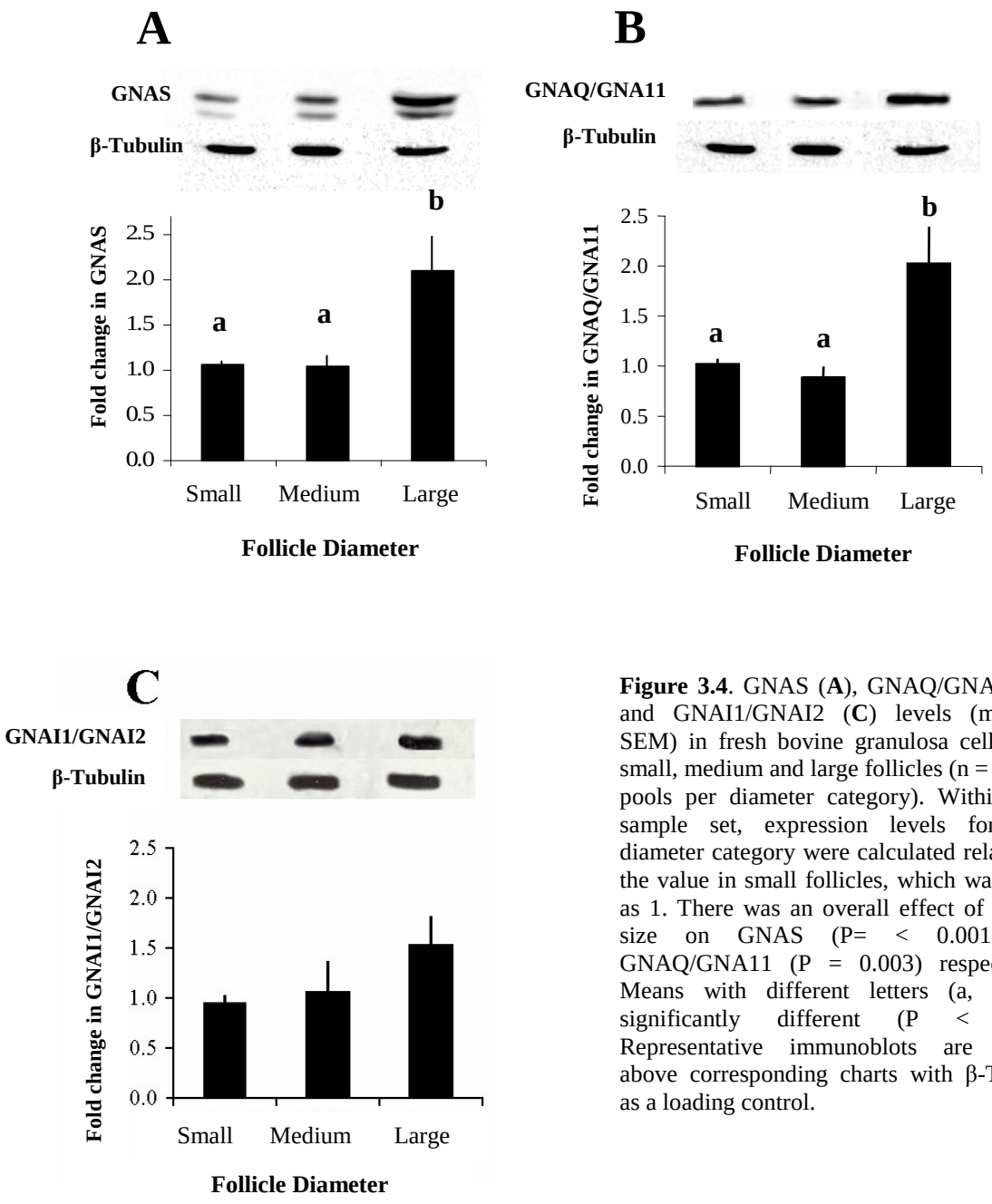


Figure 3.4. GNAS (**A**), GNAQ/GNA11 (**B**) and GNAI1/GNAI2 (**C**) levels (mean ± SEM) in fresh bovine granulosa cells from small, medium and large follicles (n = 5 to 13 pools per diameter category). Within each sample set, expression levels for each diameter category were calculated relative to the value in small follicles, which was taken as 1. There was an overall effect of follicle size on GNAS (P= < 0.001) and GNAQ/GNA11 (P = 0.003) respectively. Means with different letters (a, b) are significantly different (P < 0.05). Representative immunoblots are shown above corresponding charts with β-Tubulin as a loading control.

3.5.3 PLCB mRNA levels during follicle development

Semi-Quantitative PCR analyses of fresh bovine granulosa cells showed expression of *PLCB1*, *3* and *4* but not *PLCB2* (Figure 3.5). *PLCB3* was expressed at similar levels in granulosa cells and in positive controls (bovine brain and liver); whereas *PLCB1* and *PLCB4* were expressed at relatively lower levels in granulosa cells. Levels of PLCB4 were particularly low as they could only be detected at the highest cycle numbers using PCR.

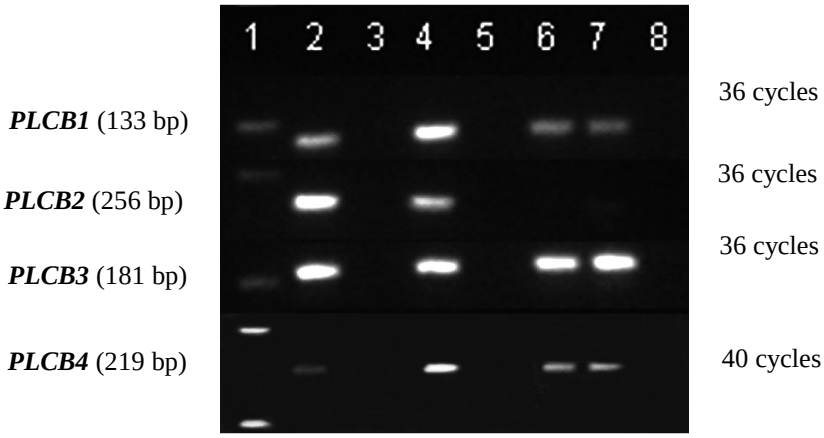


Figure 3.5. Semi-quantitative PCR of *PLCB* isoforms. Lane 1 = PCR Markers, 2 = Bovine Liver, 3 = Liver RT-, 4 = Bovine Brain, 5 = Brain RT-, 6 = Fresh Bovine Granulosa Cell pool (4-8mm follicles, n = 14), 7 = Fresh Bovine Granulosa Cell pool (15mm follicles, n = 9), 8 = Reverse Transcription negative control.

Figure 3.6 shows changes in expression of *PLCB1* (**A**) and *PLCB3* (**B**) during follicle development. There was no overall change ($P > 0.5$) in expression of *PLCB1* in granulosa cells during follicle growth whereas *PLCB3* expression increased during follicle growth ($P = 0.012$) with approximately 1.6-fold higher levels of *PLCB3* expression in granulosa cells from large than small follicles ($P < 0.05$).

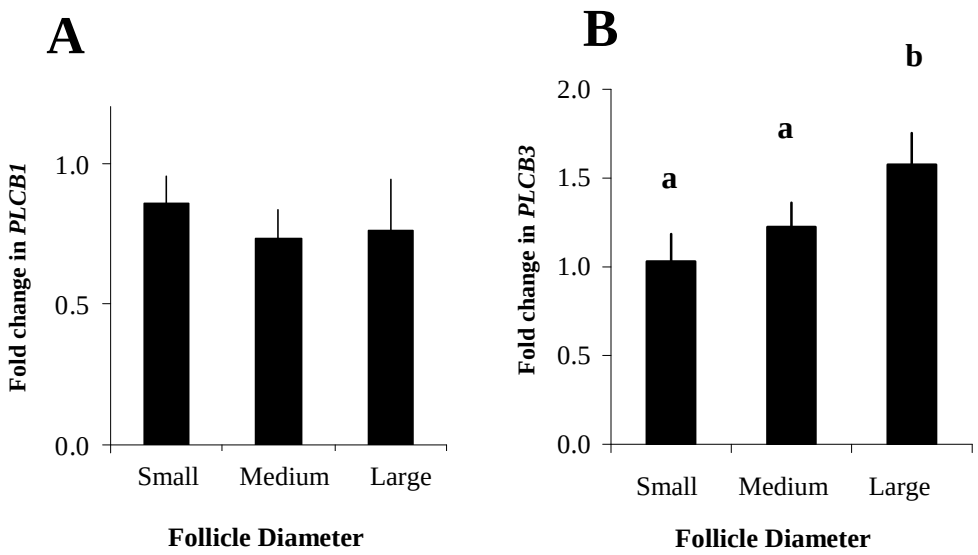


Figure 3.6. *PLCB1* (**A**), and *PLCB3* (**B**) expression (mean ± SEM) in fresh bovine granulosa cells from small, medium and large follicles (n = 5 to 13 pools per diameter category). Within each sample set, expression levels for each diameter category were calculated relative to the value in small follicles, which was taken as 1. Levels of each gene are shown relative to the value in small follicles. There was an overall effect of follicle size on *PLCB3* expression (P = 0.012). Within each graph, means with different letters (a, b) are significantly different (P < 0.05).

3.5.4 PLCB3 protein levels during follicle development

Figure 3.7 shows representative blots of PLCB3 in granulosa cells from follicles of different diameters. There was an overall effect of follicle size on PLCB3 levels ($P = 0.004$) with much higher ($P < 0.05$) levels of PLCB3 in granulosa cells from large than medium follicles (approximately 16-fold) or small follicles (19-fold).

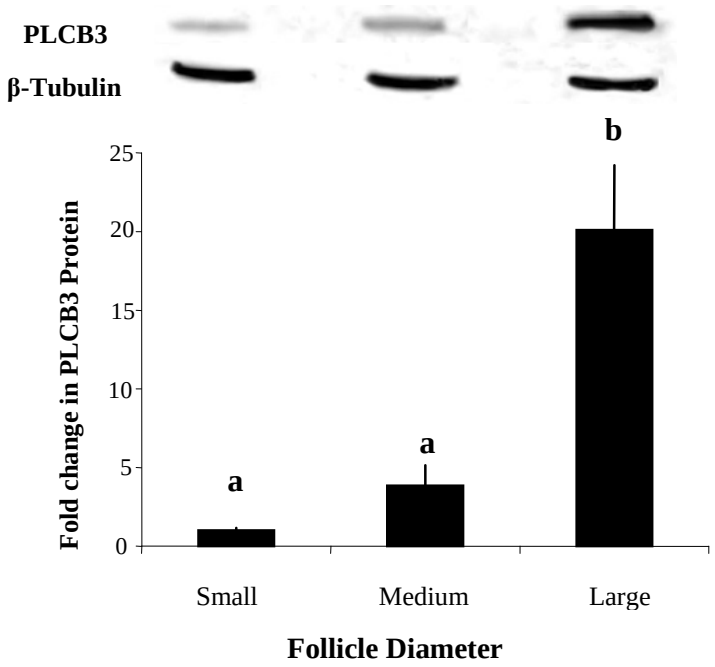
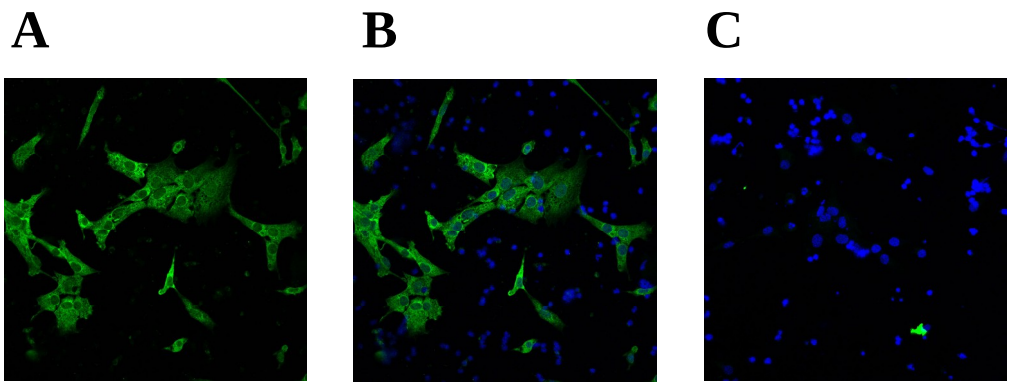


Figure 3.7. PLCB3 levels (means ± SEM) in fresh bovine granulosa cells from small, medium and large follicles (n = 5 to 13 pools per diameter category). Within each sample set, expression levels for each diameter category were calculated relative to the value in small follicles, which was taken as 1. Levels of PLCB3 are shown relative to the value in small follicles. There was an overall effect of follicle size on PLCB3 levels ($P = 0.004$). Means with different letters (a, b) are significantly different ($P < 0.05$). Representative Immunoblot is shown above chart.

3.5.5 Immunocytochemical Analysis of PLCB3 and PLCB1

Immunocytochemistry analysis of bovine granulosa cells showed predominant cytosolic localisation of PLCB3 (Figure 3.8, **A-C**). In contrast PLCB1 staining was relatively more abundant in the nucleus than in the cytoplasm of bovine granulosa cells (Figure 3.8, **D-F**).

PLCB3



PLCB1

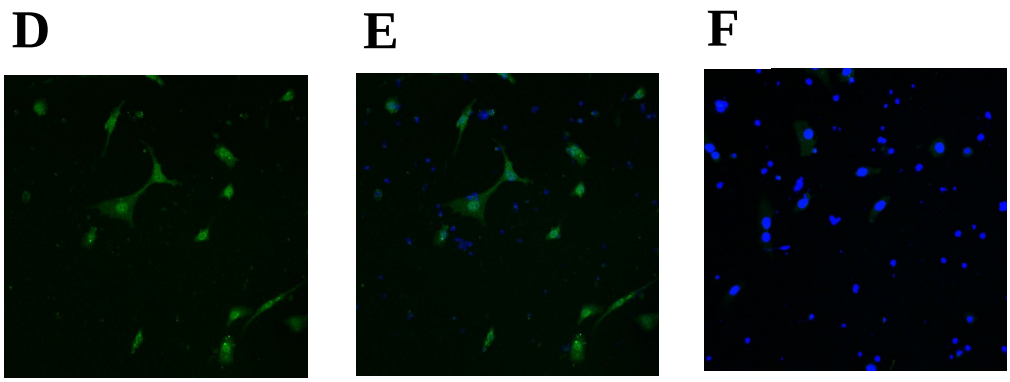


Figure 3.8. Immunofluorescence staining of PLCB3 (**A to C**) and PLCB1 (**D to F**) in cultured bovine granulosa cells (x40). Representative pictures show secondary antibody-bound FITC (green) without (A, D) or with DAPI staining (blue; B, E) and DAPI-stained negative controls (**C, F).**

3.6 Discussion

This is to our knowledge, the first study to characterise the expression of heterotrimeric Gα subunits and PLCβ subunits during follicle development in any species. The dramatic increase in mean levels of *LHR* in large follicles confirmed that visual assessment of stage of development and health of follicles was adequate. Further, there was good agreement between changes in mRNA levels and protein levels for each specific gene during follicle development. The presence of Gαs, Gαq, Gα11, and Gαi1-3 in bovine granulosa cells is consistent with a previous report on human granulosa cells (Lopez Bernal, Bellinger, Marshall et al. 1995) and also agrees with results from studies with porcine follicular membranes (Rajagopalan-Gupta, Lamm, Mukherjee et al. 1998) and bovine luteal membranes (Herrlich, Kuhn, Grosse et al. 1996).

The increase in the levels of both mRNA and protein for Gαs in granulosa cells of large follicles was in agreement with the demonstrated involvement of cAMP signalling in ovulation and luteinisation of preovulatory follicles (Conti 2002; Richards, Fitzpatrick, Clemens et al. 1995). Because these processes presumably involve a transient but massive increase in AC activity mediated by Gαs in granulosa cells in response to the elevated gonadotrophin levels of the preovulatory LH surge, an increase in levels of Gαs in preovulatory follicles would be expected.

In addition to Gαs, mRNA and protein levels of members of the Gαq/11 and Gαi families also increased in large follicles, and this was associated with a very large increase in protein levels of PLCβ3. This result is consistent with abundant experimental data suggesting that exposure of preovulatory follicles to the elevated LH concentrations of the gonadotrophin surge results in the activation of not only the Gs-AC pathway but also the Gq/Gi-PLCβ pathway. The ability to simultaneously activate different G proteins has been described for other GPCRs (Eason, Kurose, Holt et al. 1992; Laugwitz, Allgeier, Offermanns et al. 1996; Munshi, Pang, Sternweis et al. 1991). Specifically for the LHR, in vitro stimulation of rodent and pig granulosa cells with high doses of LH (100 ng/ml) resulted in a differential

increase in phosphoinositide production (Davis, Farese, & Clark 1983; Davis, Weakland, West et al. 1986) or intracellular calcium concentrations (Veldhuis & Klase 1982a; Veldhuis & Klase 1982b), both of which are products of PLCβ. In addition, in the follicle, LH receptor has been shown to couple physically and functionally not only to Gαs but also to Gαq/11, Gα13 and Gαi in porcine ovarian follicular membranes (Rajagopalan-Gupta, Lamm, Mukherjee et al. 1998; Rajagopalan-Gupta, Rasenick, & Hunzicker-Dunn 1997) and to Gαs and Gαi in luteal cells and LH-transfected cell lines (Herrlich, Kuhn, Grosse et al. 1996) . In addition it was recently shown that constitutive activation of Gαq produced granulosa cell differentiation responses similar to those induced by LH (Donadeu & Ascoli 2005). Abundant evidence also indicates that PKC, an effector of PLCβ, is involved in LH-induced granulosa cell differentiation (Donadeu & Ascoli 2005; Flores & Veldhuis 1993; Morris & Richards 1993; Morris & Richards 1995; Richards, Fitzpatrick, Clemens et al. 1995; Sen, Choudhary, Inskeep et al. 2005).

The relative contribution of Gαq and Gα11 to the observed increase in protein levels detected by the Gαq/11 antibody is not known. Although there was an overall significant effect of follicle size on Gα11 but not on Gαq mRNA levels, the mean increase in mRNA levels was similar for each subunit (1.5- and 1.6–fold, respectively), suggesting that both subunits may contribute similarly to the increase in Gαq/11 protein levels. In HeLa cells, Gα11 was 10 times more abundant than Gαq, however, both subunits contributed similarly to histamine-stimulated PLCβ activity (Krumins & Gilman 2006).

The present results indicated an increase in Gαi2 levels but not in the levels of other Gαi subunits in large follicles. In contrast to mRNA, only a minor (non-significant) increase in Gαi protein was detected because an isoform-specific antibody was not available and an antibody recognising both Gαi subtypes 1 and 2 was used. Immunocytochemistry studies in NIH 3T3 cells demonstrated that Gαi2 was located primarily at the cell surface membrane whereas Gαi3 was concentrated around intracellular membranes (Golgi apparatus in particular) and was only found on the plasma membrane when over-expressed (Hermouet, de, Spiegel et al. 1992).

In another study, it was concluded that Gαi2 was the principal mediator of the inhibition of AC in adipose cells, based on a relatively much lower efficiency of Gαi1 and Gαi3 to inhibit AC (Rudolph, Spicher, & Birnbaumer 1996). These results together with those of the current study suggest an important role of Gαi2 relative to the other two Gαi subtypes in signalling at the cell membrane level.

PLCβ isoforms are normally activated by α-subunits released upon Gq/11 activation or βγ subunits which are often derived from activation of Gi (Reviewed in Morris & Malbon 1999). Gαq/11 and Gβγ reportedly have specific affinities for different PLCβ isoforms (Reviewed by Rebecchi & Pentyala 2000), specifically, Gαq/11 may preferentially activate PLCβ1, 3 and 4. All members of the Gαq/11 family (Gαq, -11, -14 and -15/16) were shown to stimulate PLCβ1 whereas only Gα16 could activate PLCβ2 (Lee, Park, Wu et al. 1992; Park, Jhon, Kriz et al. 1992). PLCβ2 (but not PLCβ1) was also shown to be strongly activated by βγ subunits (Boyer, Waldo, & Harden 1992; Camps, Carozzi, Schnabel et al. 1992; Katz, Wu, & Simon 1992). The specificity of such G-protein activation of PLCβ isoforms remained unclear since Gαq and Gα11 were later found to stimulate PLCβ2 whilst βγ subunits were found to activate PLCβ1 (Hepler, Kozasa, Smrcka et al. 1993;Smrcka & Sternweis 1993). Although PLCβ isoforms are differentially sensitive to particular Gαq related proteins, this sensitivity is not specific and Gαq related proteins can have promiscuous associations with PLCβ isoforms (Rebecchi & Pentyala 2000; Xu, Croy, Zeng et al. 1998). It is apparent that G protein-PLCβ coupling specificities depend on the cell context. In addition, consideration should be given to the fact that the aforementioned studies were carried out in cell lines and that the observations of PLC enzymatic activities in vivo or in primary cell culture systems may be different.

In the current study, PLCβ isoforms 1 and 3 and, at very low levels, PLCβ 4, were found to be expressed in bovine granulosa cells. This is in contrast with the findings of (Lieberherr, Grosse, & Machelon 1999) who found that all four PLCβ isoforms, including PLCβ2, were expressed in porcine granulosa cells. In support of our findings, PLCβ1 and 3 isoforms are known to be widely expressed in body tissues whereas PLCB4 is less widely expressed, with highest concentrations being found in

the brain and retina (Reviewed by Rebecchi & Pentyala 2000). In contrast, PLCβ2 expression has previously been shown to be restricted to cells of hematopoetic origin (Lee, Rao, Lee et al. 1996; Li, Jiang, Xie et al. 2000). The present results are also consistent with the report by Zhong et al (2005) who found that PLCβ3 is the predominant PLCβ isoform in rat myometrial tissue (Zhong, Ku, & Sanborn 2005). Taken together these results suggest important functions of PLCβ3 in the female reproductive tract. There was no increase in PLCβ1 mRNA levels during follicle growth. In contrast, PLCβ3 expression increased during follicle growth with very high levels of PLCβ3 protein in granulosa cells of large compared to small or medium follicles.

Some PLCβ isoforms can be distinctly expressed in the cell membrane or nuclear compartments. Specifically, two alternatively spliced variants of PLCβ1, PLCB1a and PLCB1b, have been described in human, and they are preferentially expressed in the cytoplasm and nucleus respectively (Bahk, Song, Baek et al. 1998). Nuclear PLCβ1b seems to have an important role in cell differentiation processes (Faenza, Bregoli, Ramazzotti et al. 2008). Therefore, we investigated the subcellular localisation of the increase in PLCβ3 in granulosa cells by immunocytochemistry. In contrast to PLCβ1, most PLCβ3 staining was localised to the cytoplasm of granulosa cells in large follicles, consistent with an involvement of the high PLCβ3 levels in plasma membrane receptor-mediated cell differentiation. Overall, the present results on PLCβ suggest an important role of PLCβ3 in ovulation and/or luteinisation.

In summary, the results described in the current study characterise for the first time, the expression of heterotrimeric G-protein alpha subunits and PLCβ isoforms during follicle development. Results demonstrate that the granulosa cell expression of Gαs, Gα11, Gαi2 and, especially, PLCβ3 increase in dominant follicles providing support for a physiological role of the heterotrimeric G-protein/PLCβ cascade(s) in the final development and/or subsequent differentiation of dominant follicles. Further studies described in Chapter 4 investigate whether this cascade is functionally involved in the actions of VEGF in dominant follicles.

CHAPTER 4:

Cross-Talk between VEGF and heterotrimeric G-protein signalling pathways in bovine granulosa cells.

4.1 Abstract

Results in chapter 3 demonstrated an increase in the levels of Gαi, Gαq/11 and PLCB3 in bovine granulosa cells of dominant-size follicles (9-14 mm) compared to smaller follicles. The aim of this study was to investigate the potential involvement of these signalling molecules on VEGF-induced responses in granulosa cells from 9-14 mm follicles. Results showed that VEGF induced a 1.6–fold mean increase in p-ERK ($P < 0.0001$). Inhibition of Gi with PTX (pertussis toxin) completely abolished VEGF-induced p-ERK to levels that were not different from those in untreated cells ($P > 0.8$). Inhibition of Gq/11 with YM-25489 (specific Gq/11 inhibitor) partially abolished the VEGF-induced p-ERK response (1.6-fold mean increase relative to untreated controls; $P = 0.039$). LH induced a 1.6–fold increase in p-ERK1/2 ($P < 0.02$) and this response was prevented by pre-incubation with PTX ($P > 0.4$) or YM-25489 ($P > 0.5$). In contrast, similar EGF-induced phosphorylation of ERK (about 5-fold relative to controls) occurred in the absence ($P < 0.003$) or presence of PTX ($P < 0.003$) or YM-25489 ($P < 0.003$). Transfection of granulosa cells with a combination of 3 siRNA targeting *PLCB3* resulted after 48 h in approximately 40% reduction ($P < 0.005$) in levels of PLCB3 protein as determined by western blotting and immunocytochemistry. However, transfection with PLCB3 siRNAs did not have an effect ($P = > 0.7$) on phosphorylation of ERK in response to VEGF, LH or EGF in granulosa cells. In conclusion, the results of this study suggest a functional role of Gi and Gq/11 in VEGF stimulation of the ERK1/2 in bovine granulosa cells indicating that the stimulatory effects of VEGF on follicle development may be mediated, at least in part, by heterotrimeric G proteins.

4.2 Introduction

Results in Chapter 2 indicated novel and potentially important non-angiogenic roles of VEGF during the selection and ovulation of the dominant follicle. However, very little is known about the intracellular signals induced by VEGF in non-endothelial cell types. The biological activities of VEGF are mediated through its two receptor tyrosine kinases, VEGFR1 and VEGFR2. Ligand binding results in receptor phosphorylation and subsequent activation of intracellular effector pathways including Phospholipase C γ (PLC γ), Phosphatidyl-inositol 3-kinase (PI-3K) and P38 pathways (Reviewed by Cross, Dixelius, Matsumoto et al. 2003; Takahashi & Shibuya 2005).

In addition to the classical model for RTK signalling, evidence has accumulated that a number of RTKs can act through activation of heterotrimeric G-protein pathways (reviewed by Patel 2004). For example the phosphorylated epidermal growth factor receptor (EGFR) has been shown to activate the Gi/o (Johnson & Garrison 1987; Rashed & Patel 1991) and Gs (Sun, Chen, Poppleton et al. 1997; Sun, Seyer, & Patel 1995) families. Similarly, platelet derived growth factor receptor (PDGFR) signalling in smooth muscle cells seems to involve Gi, as treatment of cells with the Gi/o-specific inhibitor, pertussi toxin (PTX) results in a 40% reduction in the ability of PDGF to activate ERK (Conway, Rakhit, Pyne et al. 1999).

Zeng and associates (2003) demonstrated an involvement of G11 and Gβγ in VEGF-stimulated migration of human umbilical vein endothelial cells (HUVEC) mediated through activation of VEGFR2/RhoA (Zeng, Zhao, & Mukhopadhyay 2002a). An interaction between G11 and VEGFR2 was also found to regulate both phosphorylation of VEGFR2 and subsequent VEGF-stimulated proliferation and ERK1/2 activation in HUVEC (Zeng, Zhao, Yang et al. 2003). In contrast, VEGFR1 was found to inhibit HUVEC proliferation and this effect was mediated by a receptor interaction with Gi (Zeng, Dvorak, & Mukhopadhyay 2001a). It was concluded from these studies that signalling by VEGFR1 and VEGFR2 involves activation of Gi/o

and Gq/11, respectively. Other studies have found that Gi is involved in VEGFR1-mediated migration of macrophages (Barleon, Sozzani, Zhou et al. 1996) or basic fibroblast growth factor (bFGF)-stimulated chemotaxis in bovine aortic cells (Sa & Fox 1994). In light of present evidence, clarification of the precise roles of the Gq/11 and Gi families in VEGF signalling is clearly needed.

Not only heterotrimeric G-proteins but also its effectors have been suggested to be involved in VEGFR signalling. Specifically, there is evidence to suggest that, in addition to canonically activating PLCγ, VEGF can also interact with and activate PLCβ isoforms. Evidence of this was provided by a study using HUVEC in which stimulation with VEGF led to an increase in intracellular calcium within 40 seconds (Mukhopadhyay, Zeng, & Bhattacharya 2004). However, PLCγ phosphorylation was not reportedly detected until approximately 5 minutes after VEGF stimulation, therefore, the activities of other enzymes, namely PLCβ isoforms were tested in VEGF- stimulated endothelial cells. Results indicated no significant changes in the catalytic activities of PLCβ1, PLCβ2 or PLCγ, but an increase in the catalytic activity of PLCB3 within 20 seconds of VEGF stimulation with a peak in activity after approximately 60 seconds (Mukhopadhyay, Zeng, & Bhattacharya 2004). These findings suggested a role for PLCB3, rather than PLCγ, in VEGF-stimulated intracellular calcium release in these cells.

As indicated, evidence for a role of G proteins/PLCβ isoforms in VEGFR signalling has been obtained in studies using immortalised cell systems. In that regard, primary granulosa cell cultures provide a valuable model system that may be more physiologically relevant. Results in Chapter 3 demonstrated an increase in the expression of Gαq/11, Gαi2 and PLCB3 in pre-ovulatory size follicles in which the expression of VEGF and VEGF receptors is also highest (Kaczmarek, Schams, & Ziecik 2005; Greenaway, Connor, Pedersen et al. 2004). The temporal association between these proteins in preovulatory follicles is consistent with the functional interactions between the two signalling systems described in HUVEC. The objective of the present study was to functionally test the actual involvement of Gq/11, Gi and

PLCB3 on VEGF responses of granulosa cells by assessing the effect of specific inhibitors of Gαq/11, Gαi2 or PLCB3 on VEGF-induced phosphorylation of ERK.

4.3 Materials and Methods

4.3.1 Culture and transfection of Granulosa Cells

Granulosa cells from 9-14 mm follicles were collected and cultured as described in chapter 2. Twenty four hours after plating granulosa cells were starved overnight and then treated with VEGF (100 ng/ml), LH (100 ng/ml), EGF (100 ng/ml) or media only for 20 minutes at 37^{O}C in the presence or absence of Gi inhibitor (Pertussis Toxin, PTX, 100 ng/ml, Sigma) or a selective Gαq/11 inhibitor, YM-254890 (final concentration 100μM, gifted by Professor Robert Millar, MRC, Human Reproductive Sciences Unit, Edinburgh). PTX and YM-254890 were added to cells 24 h and 1 h before administration of treatments, respectively. After incubation cells were lysed, and protein collected for western blotting as described in chapter 2.

In another set of experiments, 500, 000 cells per well were cultured in McCoys media (Sigma, Dorset, UK) in 12 well plates (Nuclon, Nunc, Roskilde, Denmark). One day later, culture media was replaced and HiPerFect reagent (Qiagen, Sussex, UK) was used to transfect cells with 30nM of each of the three *PLCB3* siRNAs (Eurofins MWG Operon, London, UK) or 90nM of scrambled oligonulceotide (Allstars, Qiagen) according to manufacturer's protocol. Media was replaced 24 hours post transfection and plates were incubated for either 48 or 72 h before cells were lysed for protein collection or processed for immunocytochemistry. The three siRNA sequences were designed using a web based design tool, BLOCK iT™ RNAi designer (Invitrogen Corp, CA, USA) to target different regions of *PLCB3* (table 4.1) that had no homology with other *PLCB* isoforms

Table 4.1. PLCB3 siRNA sequences

PLCB3 Target	Direction	Sequence of Nucleotide
siRNA 1	Sense	5'-GGAACACCUUCUUGCGCAA-3'
siRNA 1	Antisense	5'-UUGCGCAAGAAGGUGUUCC-3'
siRNA 2	Sense	5'-GCAGCUCAUGGAUUUCAUU-3'
siRNA 2	Antisense	5'-AAUGAAAUCCAUGAGCUGC-3'
siRNA 3	Sense	5'-GCAUCCUGGUGAAGAACAA-3'
siRNA 3	Antisense	5'-UUGUUCUUCACCAGGAUGC-3'

4.3.2 Western blotting Analysis of ERK 1/2 and PLCB3

Western blotting of p-ERK/1, PLCB3 and β-Tubulin was done using procedures and reagents described in Chapter 2 and 3. For detection of total ERK, similar procedures were followed using a primary antibody (Cell Signalling Inc, Beverly, MA) at a dilution of 1:1000 and a secondary Horseradish Peroxidase (HRP)-conjugated anti-rabbit anti-immunoglobulin (Amersham Biosciences, Buckinghamshire, UK) at a dilution of. 1:10,000. The proportion of phosphorylated ERK within a protein sample was calculated by dividing the numerical value for band intensity of phosphorylated ERK by that of Total ERK.

4.3.3 Immunocytochemical Analysis of PLCB3

Immunofluorescence of cells transfected with scrambled oligonucleotide or PLCB3 siRNA for 48 hours was carried out as described in chapter 3.

4.4 Statistical Analysis

Dixon's test was used to identify suspected outlier values within data sets ($P<0.01$). These values were then excluded from subsequent analyses. The Kolmogorov-Smirnov normality test was applied to each data set to asses whether data were normally distributed. Data not normally distributed ($P<0.01$) were log transformed. Data were analysed using General Linear Model (Minitab Ltd. Coventry, UK) to determine the main effect of treatment considering each replicate as a block. For

analysis of siRNA data, the interaction of treatment by siRNA was also determined. If main effect(s) or an interaction were significant ($P<0.05$) or tended to be significant ($P<0.1$), pair-wise comparisons of means were performed using Tukey's test ($P<0.05$). Analysis of PLCB3 siRNA time-course data was done by unpaired T Test.

4.5 Results

4.5.1 Effect of PTX and YM-254890 on VEGF stimulation of p-ERK

Exposure of cultured bovine granulosa cells to VEGF (100 ng/ml) induced an increase in p-ERK relative to untreated granulosa cells ($P < 0.0001$ Figure 4.1, **A**). PTX (100 ng/ml) completely abolished the effect of VEGF treatment on p-ERK, resulting in p-ERK levels similar to those in untreated granulosa cells ($P > 0.8$). In the presence of YM-254890 (100μM), VEGF induced a 1.6 fold increase in p-ERK ($P = 0.039$), which was on average lower ($P = 0.06$) to that induced by VEGF alone (2.3-fold). Treatment with PTX or YM-254890 alone had no significant effects on p-ERK ($P > 0.5$).

Treatment with LH (100 ng/ml) induced an increase in p-ERK levels (2.5-fold; $P = 0.0115$) similar to that induced by VEGF alone (Figure 4.1, **B**). In contrast, LH failed to induced phosphorylation of ERK in the presence of either PTX ($P >0.4$) or YM-254890 ($P > 0.5$).

EGF treatment induced a 5.0-fold increase in p-ERK ($P = 0.0029$, Figure 4.1, **C**), similar to the increase induced by EGF in the presence of PTX ($P < 0.003$) or YM-254890 ($P < 0.002$).

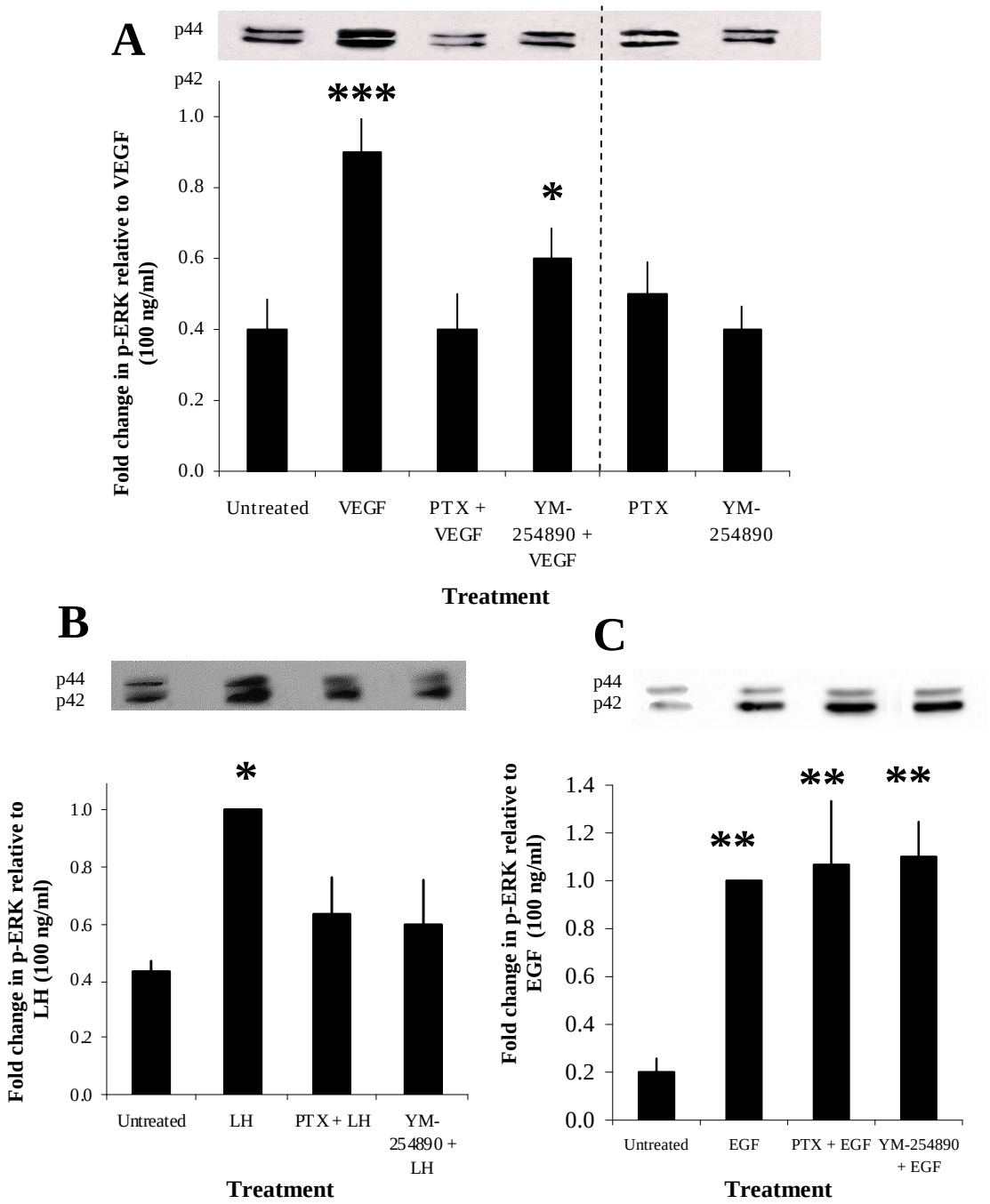


Figure 4.1. Representative immunoblots and mean (± SEM, n = 3 to 7 assays) band intensities of p-ERK in bovine granulosa cells exposed to VEGF (100 ng/ml, **A**), LH (100 ng/ml, **B**) and EGF (100 ng/ml, **C**) in the presence or absence of PTX (100 ng/ml) or YM-254890 (100µM). In each experiment, values for p-ERK were calculated relative to the value in cells treated with VEGF (A), LH (B) or EGF (C) alone. There was an overall effect of treatment on p-ERK levels in all cases (**A**: P = < 0.0001, **B**: P = 0.025 **C**: P = 0.001). *, ** or *** denotes statistical significance compared to untreated (P < 0.02, < 0.003 and < 0.0001, respectively). The same amount of protein was loaded for each sample. In A, data for inhibitor only-treated cells was analysed separately.

4.5.2 Effects of PLCB3 siRNA on PLCB3 expression

As shown in Figure 4.2, PLCB3 levels were lower in PLCB3 siRNA- than scrambled oligonucleotide-transfected cells by 48 hours (mean, 0.41 % decrease; P = 0.004) and 72 hours (mean, 0.43 % decrease; P = 0.002) after transfection.

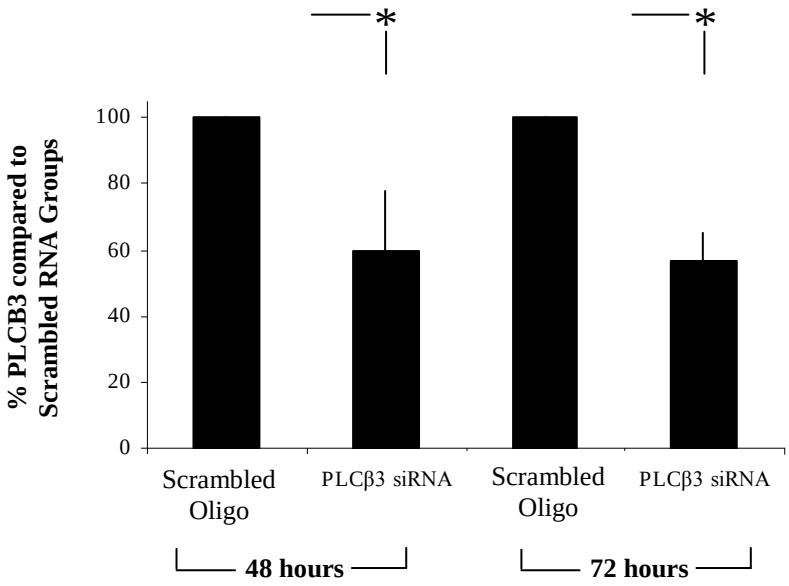


Figure 4.2. PLCB3 band intensities (Mean ± SEM) in bovine granulosa cells 48 h and 72 h after transfection with PLCB3 siRNA or scrambled oligonucleotide (90 nM, n = 4 experiments). *denotes a significant difference (P < 0.005) between treatments within a time point. PLCβ3 band intensities were calculated relative to β-Tubulin

Immunofluorescence analysis of PLCB3 48 h after transfection of granulosa cells with PLCB3 siRNA revealed a marked decrease in PLCB3 staining relative to cells transfected with scrambled oligonuclotides (Figure 4.3).

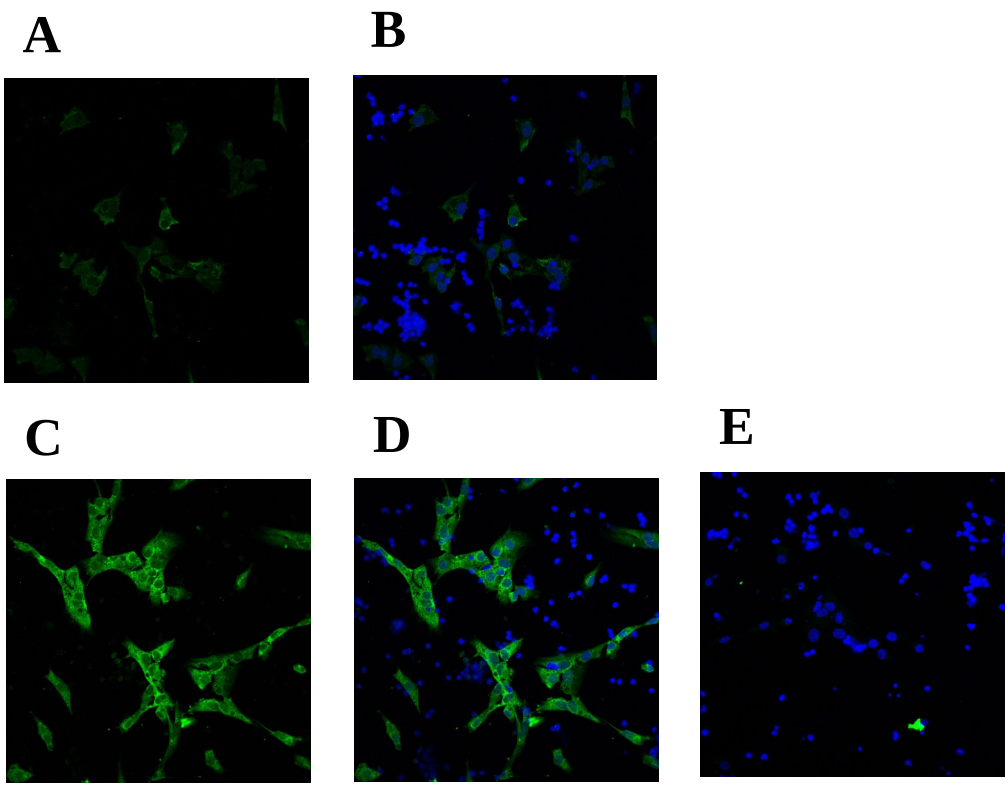


Figure 4.3. Immunodetection of PLCB3 in cultured bovine granulosa cells 48h after transfection with PLCβ3 siRNA (90nM; **A, B**) or scrambled oligonucleotide (**C, D, E**). Representative micrographs (x40) of cells incubated with PLCB3 antibody and FITC-bound secondary antibody without (**A**, **C**) or with DAPI (**B**, **D**) are shown, as well as negative control cells incubated with FITC-bound secondary antibody and stained with DAPI (**E**).

4.5.3 Effect of PLCB3 siRNA on VEGF stimulation of ERK

Treatment of granulosa cells with VEGF ($P = 0.0009$) or EGF ($P < 0.0001$), but not LH ($P > 0.9$), resulted in a significant increase in p-ERK. In all cases, p-ERK responses were not different ($P>0.7$) between scrambled oligonucleotide- and siRNA-transfected cells (figure 4.4).

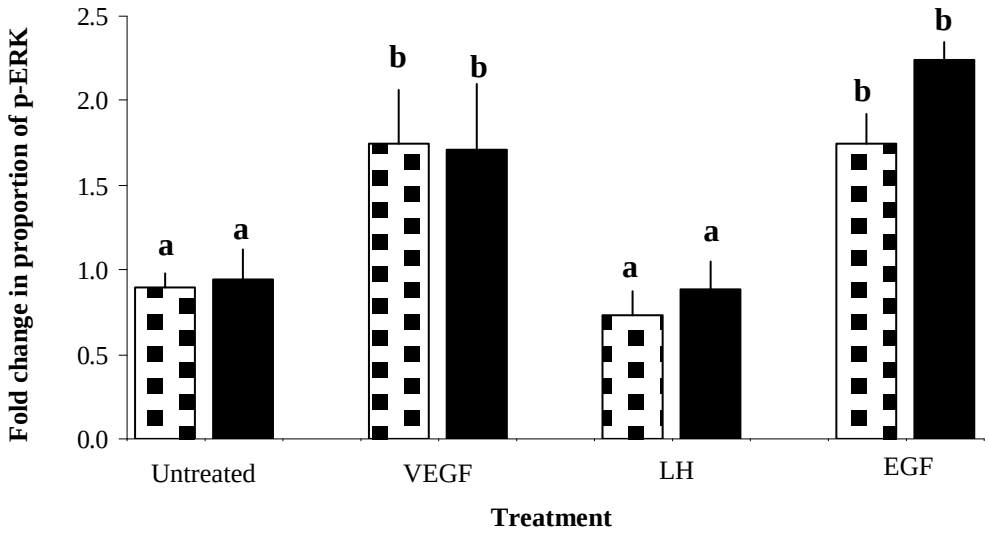


Figure 4.4. p-ERK band intensities corrected for total ERK (mean ± SEM; n =3 to 4 experiments) in bovine granulosa cells transfected with scrambled oligonucleotides, 90 nM (grey shading) or PLCB3 siRNA, 90 nM (black shading) for 48h and treated with VEGF (100 ng/ml), LH (100 ng/ml) or EGF (100 ng/ml) for 20 minutes. There was a main effect of treatment ($P < 0.0001$) but no effect of transfection ($P > 0.2$) or an interaction ($P>0.5$). Means with different letters (a, b) are significantly different ($P < 0.05$).

4.6 Discussion

The signalling mechanisms of VEGF are complex and as yet have not been fully elucidated, in particular in non-endothelial cell types. To our knowledge this study provides the first evidence of a link between the VEGF and the heterotrimeric G-protein signalling systems in the developing follicle. In the present study, p-ERK activation was used as a functional endpoint because it is a robust indicator of VEGFR activity in granulosa cells (as shown in Chapter 2), as in other cells (Kilic, Kilic, Jarve et al. 2006; Xu, Liu, Jiang et al. 2008; Gupta, Kshirsagar, Li et al. 1999) and because p-ERK plays an important role during the development of dominant follicles, most notably in mediating cell differentiation in the preovulatory follicle (Su, Nyegaard, Overgaard et al. 2006).

The results of the current study indicate for the first time that inhibition of either Gi or Gq/11 has a profound effect on the ability of VEGF to stimulate ERK phosphorylation in granulosa cells. Pre-incubation with the Gi inhibitor, PTX, completely abolished the response to VEGF, thus indicating that Gi is a requirement for effective VEGF activation of the ERK pathway in these cells. Although the Gq/11 inhibitor, YM-254890, also markedly reduced ERK phosphorylation in response to VEGF, it did not completely abolish the p-ERK response (1.6-fold increase compared to 2.3–fold increase in VEGF alone-treated cells). These results may indicate a stricter requirement of Gi than Gq/11 for VEGF induction of p-ERK1/2 in granulosa cells, however, the possibility that these differences may result from different abilities of the two inhibitors to effectively block the function of their targets should also be considered. Nevertheless, these findings are consistent with the results of Zeng et al (2003) who showed that RNAi-mediated G11 silencing resulted in inhibition of ERK1/2 phosphorylation by VEGF through VEGFR2 in HUVEC (Zeng, Zhao, Yang et al. 2003). In contrast, Gi was shown to be involved in down-regulation of HUVEC proliferation although not via VEGFR2 but via VEGFR1 (Zeng, Dvorak, & Mukhopadhyay 2001a). In other studies, EGF was shown to activate ERK in theca cells in a PTX sensitive manner (Budnik, Brunswig-Spickenheier, & Mukhopadhyay 2003), whereas the insulin receptor has been reported to associate with both Gi (Ciaraldi & Maisel 1989; Krupinski, Rajaram, Lakonishok et al. 1988; O'Brien, Houslay, Milligan et al. 1987; Rothenberg & Kahn 1988) and Gq/11 (Imamura, Vollenweider, Egawa et al. 1999; Kanzaki, Watson, Artemyev et al. 2000) to mediate the actions of insulin.

To determine the specificity of inhibition of Gq/11 and Gi on VEGF responses, the effects on LH and EGF-induced p-ERK were also analysed. LH and EGF are known to induce robust p-ERK responses in granulosa cells (Keel & Davis 1999; Salvador, Maizels, Hales et al. 2002; Woods & Johnson 2007) and this was confirmed in chapter 2 of this thesis. It has been suggested that in addition to Gs, Gi (Herrlich, Kuhn, Grosse et al. 1996; Kuhn & Gudermann 1999; Rajagopalan-Gupta, Lamm, Mukherjee et al. 1998) and Gq/11 (Rajagopalan-Gupta, Lamm, Mukherjee, et al. 1998) are involved in LH signalling. The results of the current study are in agreement

with those suggestions as LH failed to stimulate ERK phosphorylation in the presence of PTX or YM-254890.,

EGFR has been shown to couple to different heterotrimeric G-proteins depending on the type of tissue in which it is expressed. For example, in the heart EGFR has been shown to couple to Gs (Nair, Parikh, Milligan et al. 1990; Nair, Rashed, & Patel 1993), whereas in the liver this receptor is known to couple to the Gi/o family (Johnson & Garrison 1987; Liang & Garrison 1991; Rashed & Patel 1991). In the current study, neither Gi nor Gq/11 were found to be involved in EGF stimulation of p-ERK, however the possible involvement of other G-proteins can not be ruled out. Taken together, the present and previous results are consistent with a tissue-specific involvement of different G proteins in EGF signalling.

Both Gq (through Gα) and Gi (through Gβγ) are putative intracellular activators of PLCβ isoforms (Rhee 2001) and since PLCB3 is the predominant PLCβ isoform in granulosa cells of dominant-sized follicles (as demonstrated by results in chapter 3), it is reasonable to assume that interactions of VEGF with Gq and/or Gi may activate PLCB3 in these cells. Results by Mukhopadhyay et al (2004) suggested that in HUVEC, VEGF signalling was mediated via PLCB3. In the current study we tested the hypothesis that VEGF signalling in bovine granulosa cells was also mediated by PLCB3.

Although the three siRNA sequences targeting PLCB3 in this study did not completely abolish the levels of target protein after transfection (40% downregulation), in another recent study, transfection of myometrial cells with a PLCB3 shRNA reduced PLCB3 expression by only 40 to 50% and this was sufficient for functional silencing as shown by approximately 40% attenuation of an oxytocin-stimulated increase in intracellular calcium levels (Zhong, Murtazina, Phillips et al. 2008). In spite of those findings, in the present study there was no effect of PLCB3 silencing on phosphorylation of ERK induced by VEGF, as well as by LH or EGF. This initially suggested that the mechanisms of VEGF signalling in granulosa and HUVEC may differ. Unexpectedly, LH stimulation of scrambled oligonucleotide-transfected granulosa cells also did not induce any significant

response in p-ERK. In addition, it should be noted that although the response of granulosa cells to VEGF in this experiment was similar to that in earlier experiments (approximately 2-fold increase in p-ERK compared to untreated controls), the increase in p-ERK in response to EGF was substantially lower (approximately 1.7-fold relative to untreated cells) than typically observed previously (5-fold; Figures 4.1(A) and 4.1(C)). Because of the apparently low responses of cells to LH and EGF, the results of this experiment should be interpreted with caution.

The reason(s) for the reduced cell responses in the present experiment is not known. During the studies described in this thesis, ovaries collected at the abattoir usually provided not only granulosa cells but also cumulus-oocyte complexes (COCs) that were used in simultaneous studies in another laboratory. Interestingly, in this particular case, the collected COCs were reported to be of unusually poor quality and did not respond well to IVM treatments (Allison Ainslie, SAC, personal communication). These observations together with the poor responses of granulosa cells suggest that the ovaries collected for these specific experiments were, for unknown reasons, of unprecedented and unexpected poor quality, and indicate the need to repeat these tests to clarify the present results.

Although the present results did not allow any conclusions on the precise involvement of PLCB3 in VEGF signalling in granulosa cells, they did provide strong evidence that Gαi and Gαq/11 are required for VEGF-induced phosphorylation of ERK in these cells. The nature of the potential interaction between VEGFR and G proteins needs clarification. For other RTKs including EGF and IGF/IGF-I receptors a mechanism has been described whereby binding of ligand to the receptor causes membrane-associated heterotrimeric G-proteins to dissociate into α and βγ subunits and subsequent activation of downstream effector targets, similar to the mechanism, involved in GPCR signalling (Reviewed by Patel 2004). In the case of VEGFR2, it was reported that receptor activation of G11 is required for and precedes receptor tyrosine kinase activity (Zeng, Zhao, Yang et al. 2003). In support of a GPCR-like mechanism of G protein activation by RTKs, sequencing data has revealed that both EGFR and β-adrenergic receptor contains short amino

acid sequences that are consensus for Gs binding (Okamoto, Murayama, Hayashi et al. 1991; Sun, Chen, Poppleton et al. 1997). It will be essential to determine whether any Gi or Gq/11 recognition consensus sequences exist in the VEGFR2 sequence.

In addition to PLCB, several targets of Gq/11 or Gi may have a role in mediating the effects of VEGF on ERK1/2. These effects may occur though activity of either Gα or Gβγ. In HUVEC, transfection with a Gβγ-sequestering peptide did not inhibit VEGFR2 phosphorylation but did inhibit intracellular calcium mobilisation and MAPK phosphorylation which are required for HUVEC cell proliferation (Zeng, Sanyal, & Mukhopadhyay 2001). It has been proposed that VEGFR2 activation and Gβγ subunits are required for VEGF-induced stimulation of PLCγ in HUVEC cells. Further support of the involvement of Gβγ in VEGF signalling was provided by a study in zebrafish that found that silencing of Gγ2 resulted in inhibition of PLCγ/AKT-mediated angiogenic sprouting (Leung, Chen, Stauffer et al. 2006). A role of Gβγ in IGF-I-induced p-ERK has also been shown (Kuemmerle & Murthy 2001). Since Gi proteins have been described as a major source of intracellular Gβγ (Birnbaumer 2007), the dramatic effect of PTX on VEGF-induced p-ERK in the present study may be explained by a similar role of Gβγ in granulosa cells. In addition to Gβγ, Gα subunits (particularly Gαq/11) may also account for some of the effects of the G-protein inhibitors on VEGF-induced p-ERK since these two types of Gα subunit have been shown to activate directly or indirectly ERK1/2. For example, in several cell types Gαq has been shown to stimulate the ERK pathway not only through PLCβ but also via Bruton's tyrosine kinase (Btk)/PLCγ (Anger, Klintworth, Stumpf et al. 2007;Cramer, Schmenger, Heinrich et al. 2001;Lopez De, Zalduegui, Ruiz, I et al. 2006;Qiu & Kung 2000;Sanborn, Ku, Shlykov et al. 2005). Also Gαi can induce ERK1/2 signalling through Rap (Weissman, Ma, Essex, Gao, & Burstein 2004) and both Gαq and Gαi can activate PYK2 and Src which subsequently activate ERK1/2 (Dikic, Tokiwa, Lev et al. 1996; Igishi & Gutkind 1998; la Rocca, van, Daaka et al. 1997).

In summary, results of the current study indicate that Gi and Gq/11 mediate VEGF-induced activation of ERK1/2 in bovine granulosa cells. However, whether PLCB3 is

involved in VEGF signalling in these cells could not be clarified. Because ERK1/2 signalling has important roles in both proliferation and differentiation of granulosa cells, it can be concluded from the present results that Gi and Gq/11 likely mediate some of the functions of VEGF in follicles described in Chapter 2. In addition, such interactions between VEGF and heterotrimeric proteins may underlie potential cross-talk between VEGF and gonadotrophin signals in the follicles.

CHAPTER 5:

Discussion and Conclusions

Discussion and Conclusions

The studies detailed within this thesis investigated the effects and signalling mechanisms of VEGF in granulosa cells using an established bovine primary cell culture system (Gutierrez, Campbell, & Webb 1997b). The bovine has been described as a suitable model for the study of follicle development in the human as both species have similar patterns of antral follicle growth and bovine follicles are easily accessible experimentally both in vivo and ex vivo (Adams & Pierson 1995). The initial aim was to gain insight into the contribution of non-angiogenic effects of VEGF to the development of the dominant follicle. The effects of VEGF on granulosa cell proliferation, steroidogenesis and ERK1/2 activation were determined using a commercially available proliferation assay, real time PCR analysis of P450scc and western blotting, respectively, in cells from follicles of a diameter (4.0 to 8.0 mm) corresponding to the natural diameter of follicles just before they become dominant. Granulosa cells from dominant-size follicles (9.0 to 14.0 mm) were used to investigate the effects of VEGF on the periovulatory increase in COX2 (Espey & Richards 2006) using real time PCR. Although actual identities of the follicles collected in these studies were unknown, visual assessment was used to determine whether follicles were healthy (growing follicles) or atretic, as previously described (Yang & Rajamahendran 2000). The validity of this approach was confirmed by the observed increase in mean expression levels of LHR in granulosa cells from dominant-size follicles (9-14 mm; Chapter 3) consistent with these being non-atretic follicles (Bao, Garverick, Smith et al. 1997; Conti 2002). Based on previous evidence in other cell types (Mukhopadhyay, Zeng, & Bhattacharya 2004; Zeng, Dvorak, & Mukhopadhyay 2001a; Zeng, Zhao, & Mukhopadhyay 2002a), we then sought to determine the involvement of heterotrimeric G-proteins and PLCβ isoforms on the effects of VEGF in granulosa cells. Expression of heterotrimeric G-protein α subunits and PLCβ isoforms were characterised in freshly collected granulosa cells throughout antral follicle development using real-time PCR and western blotting. The specific roles of Gαq/11 and Gαi2 as well as PLCβ3 on VEGF activation of ERK1/2, a pathway that is known to mediate cell proliferative and COX2 responses (Kim, Na, Pak et al. 2008; Rodriguez-Barbero, Dorado, Velasco et al. 2006; Shaul &

Seger 2007; Souza, Shewmake, Pearson et al. 2004; Yoon & Seger 2006) were tested in granulosa cell from dominant-size follicles using specific chemical inhibitors and RNAi approaches.

The role of VEGF in follicle development has been well characterised with respect to its angiogenic effects resulting in the development of vascular networks particularly in the dominant follicle (Fraser 2006; Kaczmarek, Schams, & Ziecik 2005). Dominant follicles acquire an enriched blood supply thus receiving preferential delivery of nutrients and factors required for growth, and in this way VEGF indirectly promotes follicle selection. In recent years there has been accumulating evidence of direct effects of VEGF on proliferation, survival and migration of a wide range of non-vascular cell types (Gaudio, Barbaro, Alvaro et al. 2006; Oosthuyse, Moons, Storkebaum et al. 2001 ;Wilgus, Matthies, Radek et al. 2005; Zhu, Jin, Mao et al. 2003; Barleon, Sozzani, Zhou et al 1996; Sondell, Lundborg, & Kanje 1999c). The effects of VEGF on the development of antral follicle independent of its classical angiogenic effects have not been fully described, although limited studies have shown that VEGF can stimulate granulosa cell survival and proliferation (Grasselli, Basini, Bussolati et al. 2002; Greenaway, Connor, Pedersen et al. 2004). Particularly high levels of VEGF and VEGF receptors are found in granulosa cells of dominant follicles (Kaczmarek, Schams, & Ziecik 2005), suggesting potentially important non-endothelial roles of VEGF during the latest stages of follicle development.

Whilst *in vivo* models have undoubtedly the highest physiological relevance, separating the effects of VEGF that are due to the stimulation of blood flow in the follicle wall from those that occur through direct interaction of VEGF with non-endothelial follicular cells, such as granulosa cells, would be quite challenging and may only be reliably achieved by creating rodent models with cell-specific knockouts of the VEGF receptors. Alternatively, the use of cell culture models, with intrinsic lower complexity, offers some advantages. In that regard, the cell culture system used in the current study allowed investigation of granulosa cell responses to VEGF stimulation independently of follicular angiogenic effects.

The results of the current study suggest that VEGF is an important direct regulator of granulosa cell functions, including proliferation and periovulatory responses such as COX2 synthesis. We were interested in studying in vitro the effects of the follicle selection-associated increase in VEGF and VEGF receptors (Greenaway, Connor, Pedersen et al. 2004) on granulosa cells and for that reason we used cells from follicles with diameters encompassing those of a dominant follicle. However, VEGF may also regulate earlier stages of follicle development as evidence exists of positive effects of VEGF in pre-antral follicles in vitro. For example, Yang and Fortune (2007) recently reported that VEGF treatment of bovine cortical pieces resulted in an increased number of secondary follicles (Yang & Fortune 2007). The exact mechanisms of such stimulatory effects by VEGF in early stages of follicle growth remain unknown.

Our results (chapter 2) indicated that VEGF functionally interacts with gonadotrophins in follicles as shown by the synergistic effects of FSH and VEGF on granulosa cell proliferation and the VEGF-mediation of LH effects. The latter was indicated by the observation that inhibition of VEGFR2 using a specific inhibitor (ZM323881) significantly reduced the ability of LH to stimulate phosphorylation of ERK and completely abolished the LH-stimulated increase in COX2. This finding demonstrates for the first time that VEGF may have a primary role in mediating ovulation. Transactivation of VEGFR2 by GPCRs other than LHR has been shown. For example, stimulation of Gq/11-coupled bradykinin receptor induces tyrosine phosphorylation of VEGFR2 in cardiac endothelial cells to increase the activity of nitric oxide synthase activity (Thuringer, Maulon, & Frelin 2002). Another study showed that binding of sphingosine-1 phosphate to its Gi-coupled receptor stimulates phosphorylation of VEGFR2 (Tanimoto, Jin, & Berk 2002). In addition to VEGFR, LH has been shown to transactivate EGFR. This effect is mediated through induction of MMPs that cleave membrane bound-EGF-like growth factors which then act to rely the LH signal to COCs (Conti, Hsieh, Park et al. 2006; Park, Su, Ariga et al. 2004). Here we propose that EGF may not be the only growth factor responsible for mediation of the LH signal. Membrane-bound VEGF variants (i.e. $VEGF_{145,}$ $VEGF_{189}$ and $VEGF_{206}$) may be released in response to LH surge and act in an

autocrine and/or paracrine manner to amplify the LH signal within the follicle. Interestingly, not only LH but also EGF has been shown to up-regulate the expression of VEGF (Frank, Hubner, Breier et al. 1995; Goldman, Kim, Wong et al. 1993; Ravindranath, Wion, Brachet et al. 2001). Based on results from previous studies, LHR may transactivate VEGFR2 by stimulating the synthesis of VEGF in granulosa cells (Christenson & Stouffer 1997). Alternatively, LHR activation may stimulate the activity of intracellular ligands such as c-Src, as has shown to occur in EGFR activation (Stover, Becker, Liebetanz et al. 1995; Zwick, Hackel, Prenzel et al. 1999). Further studies are required to elucidate the precise mechanism involved in this effect.

A rational, sequential experimental approach was taken to elucidate potential roles of heterotrimeric G-proteins in these studies involving the identification of Gα subunits and PLCβ isoforms that may be functionally relevant in dominant follicles followed by actual testing of the requirement of these molecules for VEGFR signalling. This approach allowed focus on specific molecules that were likely to be physiologically relevant. The finding that Gαs, Gαq/11, Gαi and PLCβ3 were present at significantly higher levels in granulosa cells of dominant-size compared to smaller follicles indicated that these molecules may be important in relying the LH-surge signal. This conclusion is in agreement with other studies that reported that LHR in granulosa cells physically and functionally couples not only to Gs-AC but also to Gi(Gq/11)-PLCβ (Donadeu & Ascoli 2005; Herrlich, Kuhn, Grosse et al. 1996; Rajagopalan-Gupta, Rasenick, & Hunzicker-Dunn 1997; Rajagopalan-Gupta, Lamm, Mukherjee et al. 1998). The observed increase in PLCβ3 in granulosa cells of dominant-size follicles is also in agreement with results that this also was the predominant PLCβ isoform in myometrial tissue, suggesting a particularly relevant role of PLCβ3 in female reproductive tissues (Zhong, Murtazina, Phillips et al. 2008).

Results in several cell types have shown that, in addition to stimulating classical RTK-associated signalling pathways, VEGFR, as other RTKs, can mediate its effects via heterotrimeric G-protein cascades (Patel 2004; Zeng, Zhao, & Mukhopadhyay

2002a; Zeng, Zhao, & Mukhopadhyay 2002b; Zeng, Zhao, Yang et al. 2003b). We provide evidence for the first time that VEGF signal transduction can occur via Gi (most likely Gi2) and Gq/11 in granulosa cells.

Consensus binding sequences for the activation of numerous Gα subunits have been located on various RTKs, including Gαs activation sequences on the EGF receptor (Sun, Chen, Poppleton et al 1997; Sun, Seyer, & Patel 1995) and sites in insulin, β-adrenergic, muscarinic cholinergic, IGFR type 2 and α2-adrenergic receptors that are required for the activation of Gs and Gi/o (Ikezu, Okamoto, Ogata et al. 1992; Okamoto, Murayama, Hayashi, Inagaki et al. 1991; Okamoto & Nishimoto 1992). The use of bioinformatics approaches to investigate the presence of consensus sequences for binding of Gαi2 and Gαq/11 in the VEGFR2 could provide confirmation for the findings in the current studies. The identification of such sequences would in addition provide an opportunity to use site-directed mutagenesis approaches to provide a more accurate determination of the relative involvement of Gq/11 and Gi in VEGF signalling.

Our experimental approach did not enable us to distinguish whether the G protein effects on VEGF induction of p-ERK were due to Gα or Gβγ. In addition, we do not know the stoichiometric concentrations of each of the subunits in these cells. To elucidate the relative involvement of Gα and Gβγ, transfection of granulosa cells with constructs coding for βARK, which acts to bind and sequester Gβγ, or dominant negative forms of specific Gα subunits could be used. RNAi technology to target specific Gα subunits would also be useful.

At the present time we can speculate that the majority of Gβγ-mediated effects would be derived from Gi as this has been shown to be the most abundant source of Gβγ complexes in many cell types (Rebecchi & Pentyala 2000) and Gβγ is a putative intracellular activator of p-ERK, for example through PLCβ (Rhee 2001). As Gαq/11 is known to stimulate all PLCβ isoforms and according to some studies (Jhon, Lee, Park et al. 1993; Mukhopadhyay, Zeng, & Bhattacharya 2004) has a high affinity for PLCβ3, it is possible that VEGF stimulation of phosphorylation of ERK1/2 in

granulosa cells occurs in part through Gαq/11 activation of PLCβ3. Although we were unable to demonstrate an involvement of PLCβ3 in VEGF signalling mechanisms in granulosa cells, PLCβ3 levels were very high in granulosa cells of the dominant follicle and this together with evidence that VEGF Gq/11 signalling in HUVEC cells occurred via PLCβ3 further supports the argument that PLCβ3 could be involved in VEGF signalling in granulosa cells. Further work is required to elucidate the findings of the current study. One of the primary actions of Gαi2 is to reduce cAMP levels; however this subunit can activate ERK1/2 phosphorylation through pathways that include Src and Rap (Weissman, Ma, Essex et al. 2004). Although not investigated, the role of other Gα subunits such as Gαs, Gαi1, Gαi3 and Gα12/13 in mediating VEGF signals can not be ruled out. For example, Gα13 has previously been shown to be required for VEGF-induced migration of endothelial cells (Shan, Chen, Wang et al. 2006).

A working model on the intracellular mechanisms involved in VEGF-induced ERK-mediated responses in granulosa cells based on the results described in this thesis and that can be used for hypothesis-testing in future studies is shown in Figure 5.1.

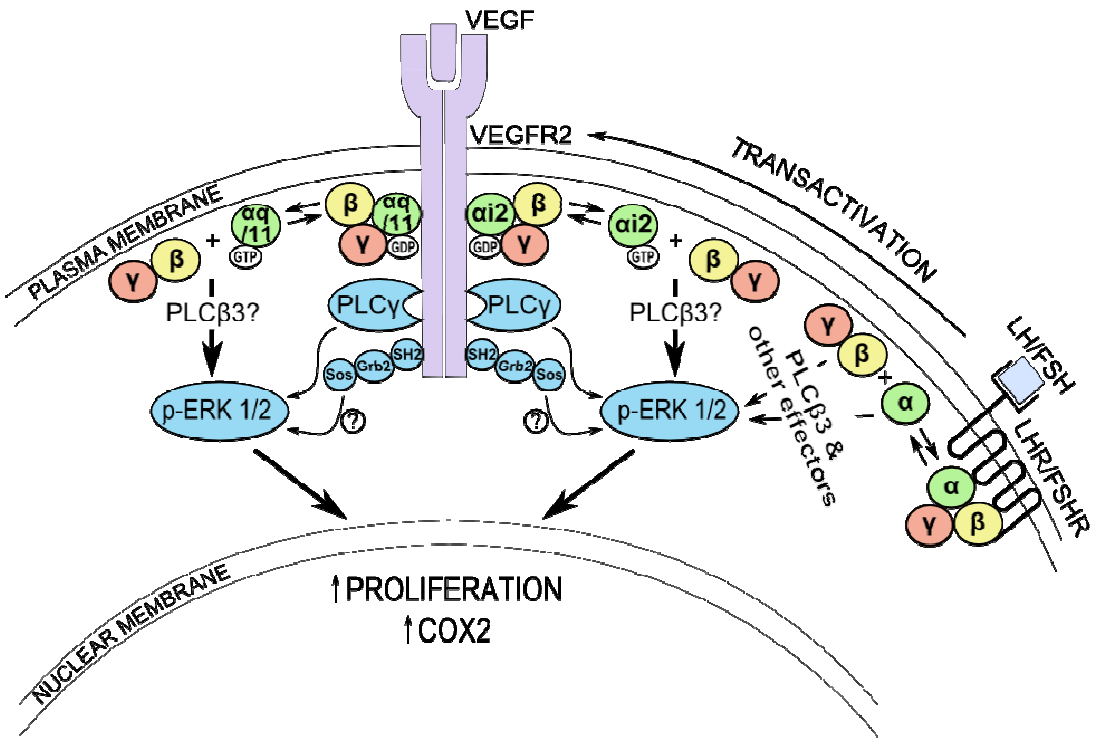


Figure 5.1. Model for VEGF-induced responses in granulosa cells from dominant follicles. The high levels of VEGF produced by granulosa cells of dominant follicles act in an autocrine/paracrine manner to stimulate cell proliferation and/or COX2 expression, depending on the stage of follicle development, two responses that involve activation of Classical pathways involved in VEGF-induced ERK activation include PLCγ and possibly the SHC/Grb2/Sos complex. Here we propose that heterotrimeric G-proteins, specifically Gi2 and Gq/11, are also required for VEGF-activation of ERK in these cells and the subsequent stimulation of cell proliferation and/or COX2 expression. This mechanism provides a functional link between VEGF and classical heterotrimeric G protein-linked trophic factors, such as gonadotrophins, in granulosa cells. Whether the effects of Gq/11 and Gi on VEGF-induced ERK are mediated by PLCβ3 remains unclear. In addition, the VEGFR also acts to mediate gonadotrophin responses in granulosa cells (such as COX2 expression) through transactivation of VEGFR by LHR (and maybe also FSHR), perhaps through a similar mechanism to that described for LHR transactivation of EGFR in ovulatory follicles, thus providing an additional cross-talk mechanism between the VEGF and gonadotrophin systems in the follicle.

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APPENDIX 1:

Seasonal effects on the response of ovarian follicles to IGF-I in mares.

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Seasonal effects on the response of ovarian follicles to IGF1 in mares

L.K. Doyle, C. O. Hogg, E. D. Watson, and F.X. Donadeu*

The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin BioCentre, Midlothian EH25 9PS, UK

Short title: Seasonal follicular response to IGF1

*Corresponding author. xavier.donadeu@ed.ac.uk, Tel. (0)131 527 4331, Fax. (0)131 440 0434

Abstract

The response of follicles to IGF1 was compared between the transition into the ovulatory season (transitional period) and the ovulatory season (ovulatory period) in eight mares using a cross-over experimental design within periods. Granulosa cells were collected from follicles 15-24 or 25-34 mm and expression of IGF1R, IGF2R, FSHR, LHCGR and PAPPA was determined by qPCR. In addition, 10 micrograms of IGF1 or vehicle were injected into the largest follicle (transitional period) or the second largest follicle (ovulatory period) of a follicular wave before the beginning of diameter deviation between the two largest follicles (mean diameters at injection, 19.2 and 20.0 mm during transitional and ovulatory periods, respectively). Follicular fluid was collected 24 h after injection for determination of Free-IGF1, IGFBP, Inhibin-A and Oestradiol levels. Granulosa cells from follicles 25-34 mm, but not follicles 15-24 mm, expressed higher levels of IGF1R (P=0.01), FSHR (P<0.007) and LHCGR (P=0.09) during the ovulatory period than during the transitional period, whereas IGF2R expression was higher in transitional than ovulatory follicles (P=0.06). Follicular IGFBP2 levels were not different (P>0.1) between periods or treatments, whereas IGFBP5 levels were higher (P<0.05) during the ovulatory period. Finally, IGF1 injection before the beginning of deviation induced a ~2 fold increase (P=0.01) in follicular Inhibin A levels during each period and did not affect Oestradiol (P>0.1). These results suggest that, as during ovulatory waves, equine follicles during transitional waves are responsive to IGF1 before the beginning of deviation and that, therefore, inadequate IGF1 responsiveness before deviation may not underlie the deficient development of dominant follicles during transition.

Keywords: Mares, Season, Transitional period, Follicle, IGF1

Introduction

The period of spring transition between the anovulatory season and the ovulatory season in the mare is characterized by renewed follicular growth (reviewed in Donadeu & Watson, 2007). During this period, follicular waves occur that can sequentially produce several large, anovulatory follicles usually in

association with irregular periods of oestrus. This makes reproductive management of these mares difficult as it is virtually impossible to predict with accuracy when ovulation will occur.

Similar to follicular waves during the ovulatory season, waves during transition are characterised by a deviation in diameter between the largest follicle and smaller follicles when the largest follicle reaches about 23 mm (Donadeu & Ginther, 2004). The largest follicle thus continues growing and becomes dominant whereas smaller follicles (subordinate follicles) cease growing and regress. However, although transitional dominant follicles can reach final diameters similar to their ovulatory counterparts they have an underdeveloped theca, are poorly vascularised, express low levels of receptors for LH (LHCGR) and have reduced capacity to produce steroids (Watson & Al-zi'abi, 2002, Acosta et al., 2004, Watson et al., 2004b). These features critically determine the anovulatory fate of these follicles. For example, adequate vascularisation as well as high intrafollicular levels of oestrogen and other growth factors enhance follicular responsiveness to gonadotropins, and this is essential to meet the increased trophic demands for the development of ovulatory-competent follicles (Ginther et al., 2003). In addition, an increase in the ability of dominant follicles to produce oestrogen at the end of the spring transition is believed to be critical for the occurrence of the LH surge that triggers the onset of the ovulatory season (Sharp et al., 1991, Sharp et al., 2001).

The reasons for the morphological, biochemical and functional deficiencies of dominant follicles during the transitional period are not clear. Low circulating LH levels and low follicular expression of LH receptor likely play a role (Donadeu & Ginther, 2002a, Watson et al., 2004b). In addition, follicular responsiveness to circulating FSH may be altered during the anovulatory season (Donadeu & Ginther, 2002a, 2003, King et al., 2008) although seasonal changes in FSH receptor (FSHR) expression in follicles have not been determined. Another trophic factor that it is likely involved is insulin-like growth factor 1 (IGF1). In ovulatory mares, IGF1 availability increases specifically in the dominant follicle before the beginning of diameter deviation (Donadeu & Ginther, 2002b, Spicer et al., 2005) where IGF1 binds to IGF receptor type 1 (IGF1R) to amplify the effects of gonadotropins on follicular cell proliferation and steroid production, among other functions (Glister et al., 2001). Expression of IGF1R has been reported

to change during antral follicle growth in sheep (Perks et al., 1995) whereas changes in IGF1R have been reported in theca cells but not granulosa cells during growth of bovine follicles (Stewart et al., 1996, Armstrong et al., 2000, Llewellyn et al., 2007). In addition to IGF1, IGF2 is found in equine follicular fluid (Bridges et al., 2002) and can bind and activate IGF1R (Spicer & Aad, 2007). IGF2 activity is negatively regulated by IGF2 binding to a second receptor, IGF2R (Delaine et al., 2007). Follicular availability of IGF1 is thought to be regulated primarily by IGF binding proteins (IGFBPs) which render IGF1 inactive (Mazerbourg et al., 2003). In ovulatory mares, levels of IGFBPs, most notably IGFBP2 and 5, decrease in the dominant follicle and this is attributable, at least in part, to the proteolytic activity of pregnancy-associated plasma protein A (PAPPA, Gerard & Monget, 1998, Gerard et al., 2004).

The decisive role of IGF1 in follicle selection and the development of ovulatory follicles in mares has been demonstrated in a series of studies by Ginther and associates who showed that injection of IGF1 into the second largest follicle of a wave at the beginning of deviation resulted in the follicle becoming dominant, rather than naturally subordinate, and eventually ovulating (Ginther et al., 2004b, Ginther et al., 2004c) whereas intrafollicular injection of IGFBP3 blocked the development of the future dominant follicle (Ginther et al., 2004a). These responses were associated with specific changes in levels of Inhibin-A and other growth factors in response to intrafollicular injection of IGF1 or IGFBP3.

In relation to seasonally anovulatory mares, Acosta et al (2004) reported lower concentrations of free IGF1 (not bound to IGFBPs) in large transitional follicles than in ovulatory follicles and Watson et al. (2004a) demonstrated that the large transitional follicles had higher levels of IGFBP2. Taken, together, these findings strongly suggest that dominant follicles during transition grow in an IGF1 deficient environment that determines the anovulatory status of transitional mares. Therefore, artificially increasing IGF1 early during development of a dominant follicle (i.e., before it begins to deviate in diameter from smaller follicles) may be potentially used to facilitate ovulation during transition, as has been reported during the ovulatory season (Ginther et al., 2004b, Ginther et al., 2004c). Such approach would only be effective if these follicles maintained their response to IGF1 during the transitional period.

The objective of this study was to investigate seasonal changes in follicular responsiveness to IGF1 in

mares by testing the hypothesis that the response to intrafollicular injection of IGF1 before the beginning of diameter deviation would be similar during transitional and ovulatory periods. This was done by determining during these two periods 1) granulosa cell expression of IGF1R as well as other genes that may have an effect on the responsiveness of cells to IGF1 namely, IGF2R, FSHR, LHCGR and PAPPA, 2) follicular levels of IGFBP2 and IGFBP5 and 3) changes in follicular Inhibin-A and oestradiol levels after an intrafollicular injection of IGF1. In testing the hypothesis, a novel experimental approach was applied by using the second largest follicle of an ovulatory wave as a model to study the responses of transitional follicles to injection of IGF1. The rationale for this was based on 1) the common anovulatory nature of these two types of follicles which is derived, at least in part, from their development in a IGF1-deficient environment and 2) the well-established biochemical and functional responses of the second largest follicle of an ovulatory wave to IGF1 injection (Ginther et al., 2004c, Ginther et al., 2005).

Materials and Methods

Animals and experimental design

Eight horse mares of mixed breeding, aged 4-16 years, body weight 400-600 kg, were kept under natural light in an open shelter and outdoor paddock in the Northern Hemisphere (55° N; Edinburgh, UK). Mares were fed alfalfa/grass hay and had free access to water and mineralized salt. All experimental procedures were carried out under the UK Home Office Animals (Scientific Procedures) Act 1986, after approval by the Ethical Review Committee, University of Edinburgh.

Beginning in February, each of the eight mares was used during two different reproductive periods, transitional (February to April) and ovulatory (June to August). Within each period, a cross-over design was used whereby each of these mares was randomly assigned once to each of two treatments, IGF1 and vehicle, leaving a 30-day rest between the two treatments. Therefore, the same mares were used within each treatment during the two periods. At the beginning of the transitional period, mares had at least one follicle > 20 mm and no detectable corpus luteum for the previous four weeks; these criteria excluded animals that were in deep anoestrus or ovulatory, respectively.

Follicle ablation, treatments and sample collection

Before each treatment during both periods, all follicles >10 mm were ablated by ultrasound-guided transvaginal aspiration of follicular contents, as described (Gastal et al., 1997), using a 17-gauge, 55-cm ovum pick-up needle (Popper & sons, Inc. New Hyde Park, NY) attached to a 5 mHz curved array transducer on an Aloka 500V ultrasound scanner (BCF Technology, Livingston, UK). This was done to eliminate follicles from previous waves and provide a uniform follicle status before the treatments across periods. During the ovulatory period, follicle ablations were always performed during mid-cycle (10 to 15 days after ovulation). In both periods, aspirates from follicles 15-24 mm and 25-34 mm were collected and separately pooled within each mare. These two diameter categories correspond to follicles before and after the beginning of diameter deviation during a follicular wave (Donadeu & Ginther, 2004). Follicle aspirates were centrifuged at 2000 × g at 4 °C for 5 min and Trizol reagent was added to the granulosa cell pellets before they were frozen at -80 °C for subsequent RNA extraction and gene expression analyses. Cell pellets with gross blood contamination were discarded.

Following ablation of all follicles >10 mm, ovaries were monitored daily with the same scanner equipped with a 5 MHz linear-array transducer (BCF Technology, Livingston, UK). At each scanning session, the diameter of follicles 10 to 15 mm was estimated by comparison with the graduation marks on the scanner screen and follicles >15 mm were measured with the electronic calipers. Follicles were measured in two planes and the average of length and width from a frozen image was taken as the actual diameter. Follicles that refilled with fluid to >15 mm were re-ablated.

During the transitional period, once the largest follicle of the post-ablation wave reached ≥18 mm, corresponding to about 1 day before the expected beginning of deviation (Donadeu & Ginther, 2004), the follicle was injected with 10 µg recombinant human IGF1 (NHPP, Torrance, CA) in a 100-µl citrate buffer or with 100 µl of citrate buffer vehicle. The dose of IGF1 was chosen based on the results of a previous dose-response study in ovulatory mares (Ginther et al., 2004c); this dose was intermediate between two doses (2.5 and 25 µg) that when injected into subordinate follicles induced responses over a 48-h period that were similar to those naturally occurring in dominant follicles. Injection was done by

ultrasound-guided transvaginal follicular puncture using an outer 20-g needle to penetrate the ovarian stroma and an inner, 25-g needle to enter the follicle, as described (Gastal et al., 1995). During the ovulatory period, the second largest follicle of the post-ablation wave was injected once the largest follicle reached 21 mm, which corresponded to an expected mean diameter for the target follicle (second largest follicle) of 18 mm (Donadeu & Ginther, 2004), comparable to the diameter of target follicles during the transitional period. During both the transitional period and the ovulatory period, follicular contents were aspirated from the target follicles 24 h after injection, followed by centrifugation and storage of the resulting supernatant at -20 C. Follicular aspirates grossly contaminated with blood were discarded (n=2 samples). Mares were scanned twice a week between treatments and the presence of a corpus luteum was used to indicate that ovulation had occurred.

Quantification of gene expression by qPCR

Total RNA from pooled granulosa cell samples for each mares was isolated using the guanidium isothiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987) and reverse-transcribed using Superscript III (Invitrogen, Paisley, UK) and random primers ($p(dN)_6$, Promega, Southampton, UK). The cDNA product was quantified using the SYBR GreenER qPCR SuperMix Universal Kit (Invitrogen) for amplicon detection and ROX as an internal reference dye in a DNA Engine Opticon 2 (MJ Research Inc. Waltham, MA, USA). Primers (Table 1) were designed based on available equine sequences or, in the case of IGF1R, based on conserved sequences in bovine, porcine and murine. Primers were designed to span two different exons within the corresponding genomic sequence and their specificity was confirmed by ensuring each product yielded the expected length on an agarose gel. PCR settings used were for all genes analysed 50C for 2 min, 95C for 10min, and 40 cycles of 95C for 15sec, 57C (IGF1R, IGF2R and PAPPA) or 52C (FSHR, LHCGR and 18S RNA) for 30sec and 72C for 45sec. The abundance of each target mRNA was calculated with Mx3000P real-time PCR system analysis software (Stratagene, La Jolla, CA) relative to a standard curve constructed from granulosa cells pooled from equine follicles of different sizes. 18S RNA was used as internal control for each of the genes analysed. Melting curves for

each sample were examined to further confirm the specificity of the qPCR product. Intra-assay CV for all genes analysed were between 0.95% and 3.44%.

IGFBP immunoblotting

Follicular fluid was assayed for protein content using the DC protein assay kit (Bio-Rad Laboratories Inc. Hercules, CA). Forty micrograms of protein (corresponding to 6 to 8 µl of a 1:10 dilution of follicular fluid) were heat-denatured in buffer containing 12%SDS, 40% glycerol, 30% -Mercaptoethanol, 300mM DTT, 120mM EDTA, 1mg/ml bromophenol blue and 375 mM TrisCl, pH 6.8 before being resolved on a 12% polyacrylamide gel. This was followed by electroblotting onto PVDF membranes for 1 h at room temperature. Membranes were then blocked for 2 h in a solution (10mM Tris, 100mM NaCl, 0.2% Tween 20) containing 5% BSA and incubated overnight with anti-bovine IGFBP2 (1:1000; 06-107, Upstate, Hampshire, UK) or anti-human IGFBP5 (1.1000, 06-110, Upstate, Hampshire, UK). Membranes were incubated with HRP-conjugated anti-rabbit anti-immunoglobulin (1:10,000; Amersham Biosciences, Buckinghamshire UK) for 1 h. After further washing, immune complexes were visualized and quantified using the Super Signal West Femto maximum sensitivity detection system (Pierce Chemical Inc., Rockford, IL) and imaged using Fluro-S Scanning System (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Densitometry analyses were done using Quantity One Analysis Software (Bio-Rad).

Free-IGF1, Inhibin-A and oestradiol immunoassays

Concentrations of free IGF1 were quantified by a sandwich-type ELISA (Diagnostic Systems Laboratories, Webster, TX) adapted for use with mare follicular fluid (Donadeu & Ginther, 2002b). Samples were used undiluted. Antisera cross-reactivity with IGF2, insulin, or IGFBPs was not found by the manufacturer. Intra-assay CV was 6.5%, and assay sensitivity was 25.0 pg/ml. For all assays, sensitivity was considered as 2 standard deviations above the mean OD of the zero standard corrected for sample dilution.

Concentrations of Inhibir-A in the follicular fluid were determined with a sandwich ELISA kit (Diagnostic Systems Laboratories). The kit was developed for use with human samples and was adapted and validated for use with equine follicular fluic in our laboratory. Serial dilutions (1:100 to 1:100000) of a pool of equine follicular fluid in the provided assay diluent resulted in a displacement curve that was parallel to the standard curve provided (0 to 1 ng/ml). A working dilution of 1 1000 was usec for assaying the follicular-fluid samples. This was chosen based on the dilution of pooled follicular fluid that resulted in an optical density (OD) that was central to the range of the sandard curve. According to the manufacturer, there was no significant cross-reactivity of the assay with Inhibin-B, pro- C inhibin or Acivin-A and -B. The intra-assay CV was 6.7%. and the sensitivity was 19.7 ng/ml.

Oestradiol levels in follicular fluid were measured by radioimmunoassay as described (England et al., 1981) without previous extraction of samples. The intra-assay CV was 9.6% and the sensitivity was 5.0 pg/ml.

Statistical Analyses

Dixon's Q-test was used to identify outliers using P<0.05 as a cut-off value to exclude a sample from subsequent analysis. Data for each endpoint were log-transformed before analyses whenever a Kolmogorov-Smirnoff test (significance level=0.01) indicated lack of normality. For all endpoints, main effects (Period, Follicle size and/or Treatment) and their interactions were analyzed with the General Linear Model (Minitab 15) using a split-plot design with Mare as block. To rule out any carry-over effect derived from the repeated use of mares within each period, an additional main effect included in the analyses was whether a treatment was first or second during a period. When a main effect was significant or approached significance, Tukey's test was used to locate differences between means. A probability of <0.05 indicated that a difference was significant and probabilities between >0.05 and <0.1 indicated a difference approaching significance.

Results

The first ovulation of the year occurred between 11 April and 24 May (median date, 29 April) which coincides with the expected onset of the ovulatory season in this latitude (Watson et al., 2004a).

Gene expression in granulosa cells

Granulosa cell pellets for gene expression analyses were obtained from a total of 12 follicle ablation sessions during the transitional period and 14 sessions during the ovulatory period. Between 4 and 7 pools (one to four follicles per pool) were obtained within the 15-24 mm and 25-34 mm categories during each period. Mean diameter of follicles included in a pool during transitional and ovulatory periods were 19.2 ± 2.0 and 16.9 ± 1.0 mm, respectively, for the 15-24 mm category, and 29.7 ± 0.3 and 31.7 ± 1.1 mm for the 25-34 mm category. Mean diameters were not different (P>0.1) between periods within each category.

Results of qPCR analyses are shown in Figure 1. Although there were no main effects (P>0.4) of Period or Follicle Size for IGF1R expression, there was a significant (P=0.03) interaction due to 6-fold higher (P=0.01) mean levels of IGF1R mRNA in 25-34 mm follicles during the ovulatory period than during the transitional period but no differences (P>0.5) between 15-24 mm follicles during the two periods. Analysis within period indicated that during the ovulatory period 25-34 mm follicles had greater (P=0.04) IGF1R mRNA levels than 15-24 mm follicles. Although there was no effect of Follicle Size or an interaction (P>0.2) for IGF2R expression, the effect of Period tended to be significant (P=0.06), with higher mean expression levels during the transitional period than during the ovulatory period. As for IGF1R, there were no main effects (P>0.2) of Period or Follicle Size for FSHR expression but there was an interaction (P=0.007) which was due to higher levels (~5 fold) in 25-34 mm follicles during the ovulatory period than during the transitional period. In addition, during the transitional period 15-24 mm follicles had greater (P=0.024) levels of FSHR mRNA than 25-34 mm follicles. For LHCGR expression, the interaction between Period and Follicle Size approached significance (P=0.09) due to higher mean expression in 25-34 mm follicles during the ovulatory period than during the transitional period. There were no overall main effects or an interaction (P>0.2) for PAPPA expression, however, analysis of

expression levels within the ovulatory period indicated a tendency (P=0.08) for higher PAPPA mRNA levels in 25-34 than 15-24 mm follicles.

Follicular responses to IGF1

Data from a total of seven mares were removed from analyses after follicular injections for the following reasons; target follicle could not be identified with total certainty (3 mares), failed intrafollicular injection (1 mare), follicle collapsed after injection with vehicle (1 mare) and follicular fluid was grossly contaminated with blood (2 mares). As a result, data from five to seven mares were available for anayses within each treatment and period.

Diameters of target follicles on the day of injection were not affected by Period (P>0.1) and were similar (P>0.1) between vehicle- and IGF1-treated mares during the transitional period (largest follicle, 19.2 ± 0.3 and 19.1 ± 0.5 mm) and the ovulatory period (second largest follicle, 20.4 ± 1.2 and 19.8 ± 0.3 mm, respectively). Changes in diameter during the first 24 h after injection of vehicle or IGF1 were 0.6 ± 1.3 and -0.3 ± 1.4 mm, respectively, for the largest follicle during the transitional period, and -2.0 ± 2.1 and -1.6 ± 1.5 mm for the second largest follicle during the ovulatory period. These changes were not affected (P>0.1) by Period, Treatment or their interaction.

As expected free IGF1 concentrations in follicular fluid (Figure 2a) increased after IGF1 injection (main effect of Treatment, P=0.01) but were not affected by Period or the interaction of Treatment x Period (P>0.1). Determination of relative IGFBP2 and IGFBP5 levels in follicular fluid by immunoblotting revealed bands that were 32-36 Kd (Figure 2b) and 25-29 Kd (Figure 2c), respectively, which is consistent with previous reports using Western ligand blotting in equine follicular fluid (Gerard & Monget, 1998, Bridges et al., 2002, Watson et al., 2004a). Follicular IGFBP2 levels were not affected (P>0.1) by Period, Treatment or Period x Treatment but this interaction was significant (P<0.03) for levels of IGFBP5 due to higher levels in vehicle-treated mares during the ovulatory period than in IGF1-treated mares during the ovulatory period (P<0.05) and vehicle-treated mares during the transitional

period (P<0.05).

Follicular Inhibin-A concentrations (Figure 3a) were affected by Treatment (P=0.01) but no by Period or Treatment x Period (P>0.1). Injection of IGF1 irduced mean increases (P=0.05) in Inhibir-A levels of 2.1- and 2.2-fold during the transitional and ovulatory periods, respectively.

Finally, although there was no overall effect (P>0.4) of Treatment or an interaction, oestradiol levels in follicular fluid were affected by Period (P=0.05) resulting in mean higher oestradiol levels (1.8-fold) during the ovulatory period than during the transitional period (Figure 3b).

Discussion

This study used two complementary approaches in mares to investigate for the first time in vivo seasonal changes in follicular responses to IGF1, namely, 1) analyses of follicular IGF1R expression and IGFBP levels, both of which are major determinants of responsiveness to IGF1, and 2) quantification of actual follicle responses to intrafollicular injection of 10 µg IGF1, as indicated by changes in follicular Inhibin-A and oestradiol levels. The use of the second largest follicle of an ovulatory wave as a reference to study the responses of transitional follicles is novel. Unlike the largest follicle of ovulatory waves (future dominant follicle), the second largest follicle is not exposed to an increase in follicular IGF1 at the beginning of deviation (Donadeu & Ginther, 2002b) which contributes to its inability to become ovulatory (Ginther et al., 2004b, Ginther et al., 2004c). Transitional follicles develop in a similar IGF1-deficient environment after deviation resulting in developmental deficiencies that prevent them from acquiring the ability to ovulate (Acosta et al., 2004, Watson et al., 2004a). The present study demonstrates that experimental comparisons between these two types of follicles can be useful to understand the biological mechanisms behind the anovulatory nature of transitional follicles and opens the way for the potential use of this comparative approach in future studies.

To our knowledge changes in IGF receptor expression during follicular development have not previously been reported in the horse. IGF1R expression increased with follicle size during the ovulatory period but not during the transitional period, and this resulted in higher expression levels in 25-34 mm

follicles during the ovulatory period than during the transitional period but similar levels in 15-24 mm follicles between the two periods. This result is consistent with the essential role of IGF1 in the selection and subsequent development of ovulatory follicles (reviewed in Beg & Ginther, 2006) and also with the causative role of deficient IGF1 stimulation in the failure of transitional follicles to develop characteristics of ovulatory follicles (reviewed in Donadeu & Watson, 2007). The present results in mares are in contrast with some of the previous studies in which IGF1R expression in granulosa cells did not change (Armstrong et al., 2000, Liu et al., 2000, Hastie & Haresign, 2006, Llewellyn et al., 2007) or decreased (Perks et al., 1995) during growth of antral follicles in cows and pigs or between seasons in sheep. Taken together, these results highlight species-specific differences in the pattern of IGF1R expression during follicle growth, however, it must also be recognised that quantification of mRNA levels in the present and previous studies may not adequately reflect the actual levels of functional IGF1R in follicles.

The expression levels of IGF2R, FSHR and LHCGR in granulosa cells were determined because the responses of follicular cells to IGF1 may depend, at least in part, on their concurrent responsiveness to IGF2 (Spicer & Aad, 2007) and gonadotropins (Glister et al., 2001). IGF2 is naturally produced in equine follicular cells (Davidson et al., 2002, Watson et al., 2004a) and IGF2 expression is lower during the transitional period than during the ovulatory season (Watson et al., 2004a). IGF2 has been shown to promote bovine granulosa cell proliferation and steroidogenesis by binding to IGF1R (Spicer & Aad, 2007). In contrast, IGF2 binding to IGF2R results in the inactivation of IGF2 (Delaine et al., 2007). In this study, expression of IGF2R did not change significantly with follicle size but was higher during the transitional period than during the ovulatory period. Considering the role of IGF2R in regulating IGF2 bioavailability in the follicle, the present results suggest that the ability of IGF2 to activate IGF1R may be impaired in transitional follicles relative to ovulatory follicles. Although the effects of IGF1 injection on IGF2R were not determined in this study, IGF2R expression in bovine granulosa cells has been shown to decrease in response to IGF1 (Spicer & Aad, 2007).

The effects of season on follicular FSHR expression in horses have not been previously reported. In an earlier study, Fay and Douglas (1986) failed to demonstrate changes in FSH binding to follicle walls

during the oestrus cycle and those findings are consistent with the absence of significant changes in FSHR expression between follicles 15-24 mm and 25-34 mm during the ovulatory period in the present study. In contrast, FSHR expression decreased as transitional follicles grew above 24 mm. The reason for this is not known but it does bring the possibility that a reduced responsiveness not only to LH but also to FSH may be involved in the failure of these follicles to acquire ovulatory capacity. The higher LHCGR expression in dominant-size follicles during the ovulatory period than during the transitional period is consistent with previous results (Watson et al., 2004b). Although not critically tested in the present study, reduced LHCGR expression in transitional follicles is likely mediated, at least in part, by low levels of IGF1 in these follicles (Hirakawa et al., 1999). Overall, the results on receptor expression in this study indicated that follicles 15-24 mm have similar responsiveness to trophic stimuli, namely, IGF1, FSH and LH, during transitional and ovulatory periods. Further analyses of IGF1 responses of these follicles between periods were then performed by analysing follicular IGFBP levels and changes in Inhibin-A and oestradiol in response to intrafollicular IGF1 administration.

There was no effect of Period on intrafollicular levels of free IGF1 and this is consistent with results indicating that differences in intrafollicular levels of free IGF1 between transitional and ovulatory waves do not occur before the beginning of deviation (Acosta et al., 2004, Donadeu, 2006). During the two periods, mean follicular levels of free IGF1 in mares injected with IGF1 or vehicle were lower than those reported in some of the previous equine studies (Donadeu & Ginther, 2002, Ginther et al., 2004a) but were comparable to natural levels in dominant and largest subordinate follicles, respectively, at the beginning of deviation during ovulatory waves in another study (Ginther et al., 2005). The magnitude of the increase in intrafollicular free IGF1 after injection of 10 ug of IGF1 (average over the periods, 23.2-fold over saline-treated follicles) was consistent with results of previous IGF1 injection studies in ovulatory mares (Ginther et al., 2004c, Ginther et al., 2005).

In the present study, vehicle-treated follicles were used to assess seasonal differences in the levels of IGFBP2 and 5, both of which critically contribute to the differential increase in IGF availability to the dominant follicle in ovulatory mares (Gerard & Monget, 1998, Bridges et al., 2002, Donadeu & Ginther,

2002b). The follicular puncture procedure used in this study has by itself no detectable effects on levels of follicular factors, including IGFBPs (Ginther et al., 2004c). Follicular IGFBP2 levels were similar between the transitional period (mean diameter of the largest follicle at the time of sampling, 19.5 mm) and the ovulatory period (mean diameter of the second largest follicle, 18.6). In agreement with the present results, IGFBP2 levels were not different between 13-mm follicles collected during the transitional period and the ovulatory season (Donadeu, 2006). In contrast, large follicles (>30 mm) had relatively higher IGFBP2 levels during the transitional period (Watson et al., 2004a). Taken together, these findings suggest that, in contrast to the ovulatory season (Gerard & Monget, 1998, Bridges et al., 2002), a decrease in follicular IGFBP2 levels in dominant follicles does not occur during the transitional period. A tendency for greater mean expression levels of PAPPA, an IGFBP protease (Gerard et al., 2004), in follicles 25-34 mm than in follicles 15-24 mm during the ovulatory period but not during the transitional period in this study are consistent with that conclusion. The lack of an effect of IGF1 injection on follicular IGFBP2 levels is in agreement with results by Ginther and collaborators (Ginther et al., 2004c, Ginther et al., 2005) who showed that a much higher dose of IGF1 than the one used in the present study is needed to induce any detectable changes in follicular levels of IGFBP2.

The effects of season or IGF1 on follicular IGFBP5 levels in mares have not been reported before. The reason for higher levels of IGFBP5 during the ovulatory period (second largest follicle) than during the transitional period (largest follicle) in vehicle-treated mares is unknown. IGFBP5 levels (as well as levels of IGFBP2 and IGFBP4) have been shown to increase in subordinate follicles of ovulatory mares (Gerard & Monget, 1998, Donadeu & Ginther, 2002b). Based on results in cattle and horses, changes in follicular-fluid levels of IGFBP5 and IGFBP4 during follicular waves seem to occur before the beginning of diameter deviation (reviewed in Beg & Ginther, 2006). In contrast, changes in follicular IGFBP2 levels are only detectable after deviation. Therefore, it is conceivable that in the present study IGFBP5 levels (but not IGFBP2 levels) in the target follicle of the ovulatory period (second largest follicle) were already increasing when follicular fluid was collected for analyses. Another observation in the present study was that IGFBP5 levels were lower in IGF1- than vehicle-injected follicles during the ovulatory period.

Follicular levels of IGF1 and IGFBP5 are negatively correlated during follicular growth in mares (Bridges et al., 2002). Considering that, together with the apparent early involvement of IGFBP5, compared to IGFBP2, during follicle selection (Beg & Ginther, 2006), it is plausible that in the present study IGFBP5 levels in the second largest follicle during the ovulatory period decreased in response to exogenous IGF1, a possibility that warrants further testing. Overall, these results on IGFBPs suggest that follicular IGFBP activity may be comparable during the transitional and ovulatory periods (based on IGFBP2 levels) or lower during the transitional period (based on IGFBP5). Although the relative contribution of IGFBP2 and IGFBP5 to regulation of follicular IGF1 activity in mares has not been directly determined, previous results using Western ligand blotting suggest that follicular IGFBP2 is predominant over IGFBP4 and IGFBP5 during both the ovulatory and transitional periods (Gerard & Monget, 1998, Watson et al., 2004a). Considering this, overall IGFBP activity within follicles before the beginning of deviation is likely to be similar during transitional waves and ovulatory waves and this is consistent with similar levels of free IGF1 after IGF1 injection during the two periods in this study.

Inhibin-A levels have been consistently shown to increase after IGF1 injection into the second largest follicle of ovulatory waves (Ginther et al., 2004c, Ginther et al. 2005) as well as in the largest follicle during natural follicle selection (Donadeu & Ginther, 2002b). The mean increase in intrafollicular Inhibin-A induced by 10 µg of IGF1 during the ovulatory period (about 2-fold relative to vehicle-treated controls) was comparable to the increase reported after IGF1 injection into the second largest follicle in a previous study (Ginther et al., 2004c). More importantly, the Inhibin-A responses were similar during transitional and ovulatory periods and this was consistent with the absence of detectable differences in expression of IGF1R and follicular levels of IGFBP2 before the beginning of deviation between the two periods.

The differences in follicular oestradiol levels between periods in the present study indicate that deficient follicular steroidogenesis during transition is not limited to late dominant follicles (Davis & Sharp, 1991, Watson et al., 2002, Watson et al., 2004b) but it apparently also involves follicles before the beginning of deviation. Since IGF1 is known to simulate steroidogenesis in vitro (Glister et al., 2001)

and there is a temporal association between reduced IGF1 activity and low oestradiol levels during transition (reviewed in Donadeu & Watson, 2007). we wished to examine whether experimentally increasing intrafollicular IGF1 activity before the beginning of deviation during the transit onal period would lead to ar stimulation of oestradiol production. The absence of an oestradiol response to IGF1 in that regard is similar to results after intrafollicular IGF1 injection before the beginning of deviation in ovulatory mares (Ginther et al., 2004c; Ginther et al., 2005) and is consistent with results in vitro showing that the ability of granulosa cells to respond with an increase in oestradiol may depend on the size of the follicles (Davidson et al., 2002). The present result indicates that, as during the ovulatory season, IGF1 may not acutely regulate oestradiol during the transitional period. It is also possible that a higher dose of IGF1 than the one used in this study was necessary to adequately stimulate not only granulosa cells but also theca cells which is likely required to efficiently stimulate the natural increase in follicular production of oestradiol at the end of the transitional period (Watson et al., 2004b). Nonetheless, the present results together with those of previous studies (Donadeu & Watson, 2007) strongly suggest that the distinct increase in production of oestradiol by dominant follicles at the end of the transitional period involves not only an increase in IGF1 bioactivity but also an increase in the responsiveness of follicles to IGF1 and gonadotropins (and likely other trophic factors).

In summary, expression of IGF1R, as well as FSHR and LHCGR, in granulosa cells from follicles 15-24 mm was similar during the transitional period and the ovulatory period, whereas expression of each of these receptors in follicles 25-34 mm was lower during the transitional period. IGF2R expression tended to be higher during the transitional period than during the ovulatory period, regardless of follicle diameter. In addition, follicular levels of IGFBP2 before the beginning of deviation were similar during the two periods whereas levels of IGFBP5 were lower during the transitional period. Injection of IGF1 into the largest follicle of a transitional wave before the beginning of deviation induced a ~2 fold mean increase in follicular Inhibin-A levels, similar to that induced after IGF1 injection into the second largest follicle of a wave during the ovulatory period. Finally, follicular oestradiol levels did not respond to IGF1 during any of the two periods. Taken together, these results indicate that follicular responsiveness to IGF1

before the beginning of deviation is comparable during transitional waves and ovulatory waves and, therefore, this is likely not a primary cause of the deficient development of dominant follicles during transition.

Declarations of interest, funding and acknowledgements

Declarations of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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Figure Legends

Figure 1. Relative mRNA levels (mean ± SEM) of (a) IGF1R, (b) IGF2R, (c) FSHR, (d) LHCGR and (e) PAPPA in granulosa cells collected from pools of 15-24 mm and 25-34 mm follicles (one to four follicles per pool) during the transitional period (dashed line, open circle; n=7 and 4 pools, respectively) and the ovulatory period (solid line, closed circle; n=6 and 6 pools). The interaction between Follicle size and Period was significant for IGF1R (P=0.03) and FSHR (P=0.007) and approached significance for

LHCGR (P=0.09), whereas an effect of Period for IGF2R tended to be significant (P=0.06). An asterisk indicates a significant difference (P<0.05) between periods within follicle size. Different letters (a,b) indicate a significant difference (P<0.05) between follicles sizes within period.

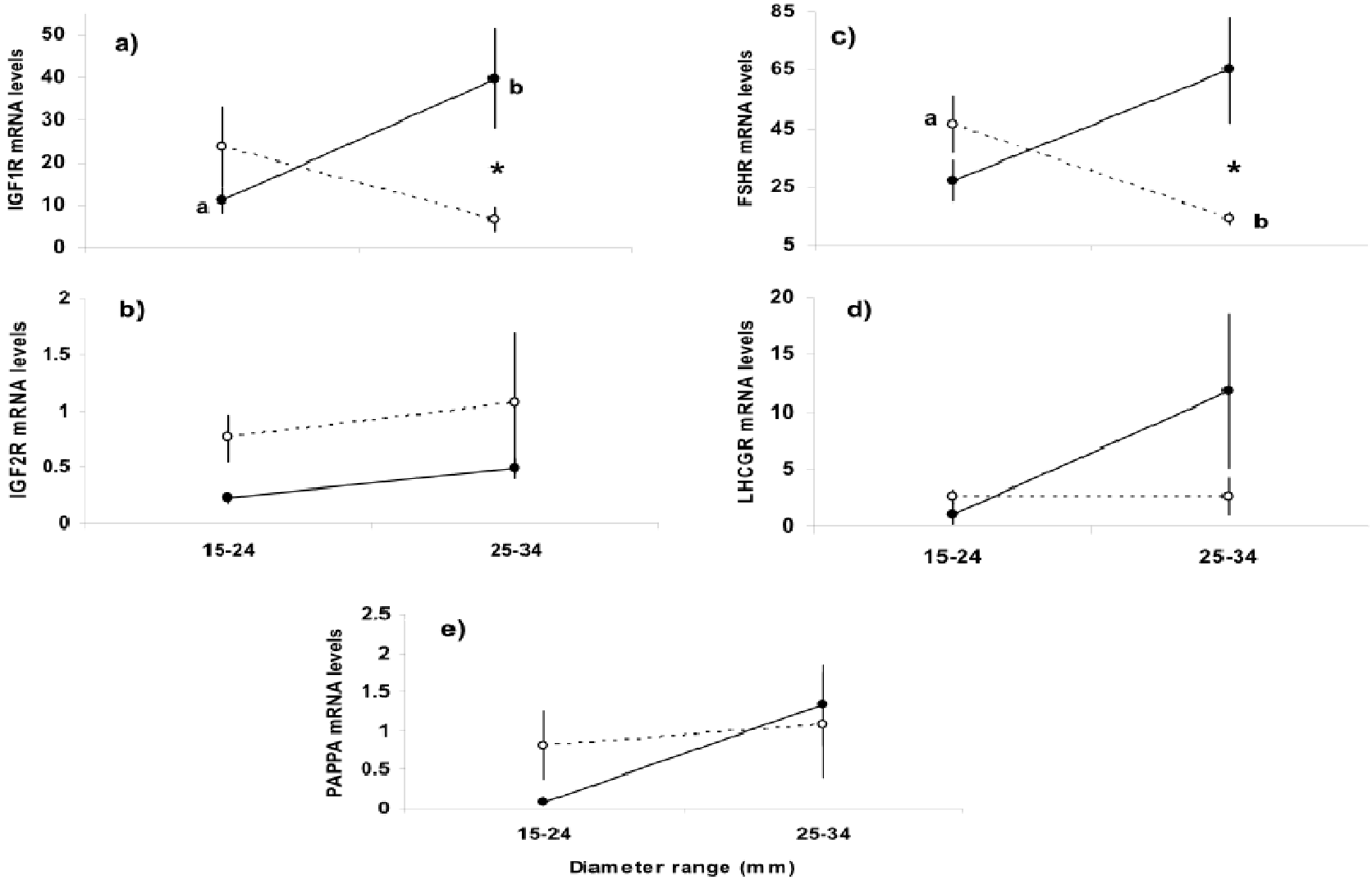
Figure 2. Relative follicular-fluid levels (mean ± SEM) of (a) free IGF1, (b) IGFBP2 and (c) IGFBP5 in mares (n=5 to 6/group) 24 h after injection of vehicle (white bars) or 10 µg IGF1 (black bars) into the largest follicle of a wave during the transitional period or the second largest follicle during the ovulatory period. There was an effect of Treatment for free IGF1 (P=0.01). There were no main effects or an interaction for IGFBP2 (P>0.1) but there was an interaction of Period x Treatment (P=0.027) for IGFBP5. Means with different letters (a,b) are different (P<0.05). Representative immunoblots for each IGFBP are shown above the corresponding graph.

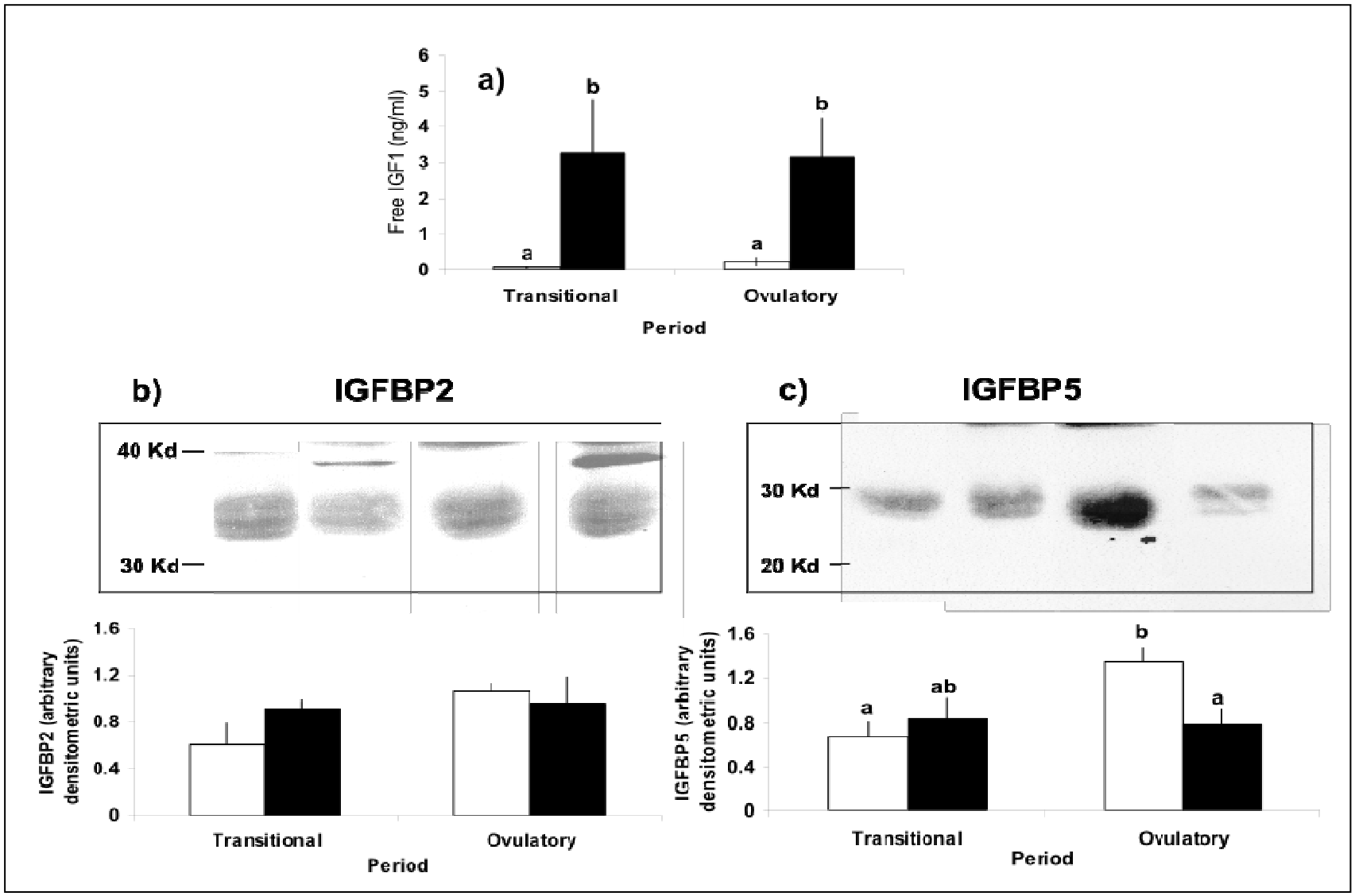
Figure 3. Mean (± SEM) follicular-fluid concentrations of a) Inhibin-A and b) Oestradiol in mares (n=5 to 7/group) 24 h after injection of vehicle (white bars) or 10 µg IGF1 (black bars) into the largest follicle of a wave during the transitional period or the second largest follicle during the ovulatory period. There were significant effects of Treatment for Inhibin-A (P=0.07) and of Period for Oestradiol (P=0.05). Means within Periods with different letters (a,b) are different (P<0.05).

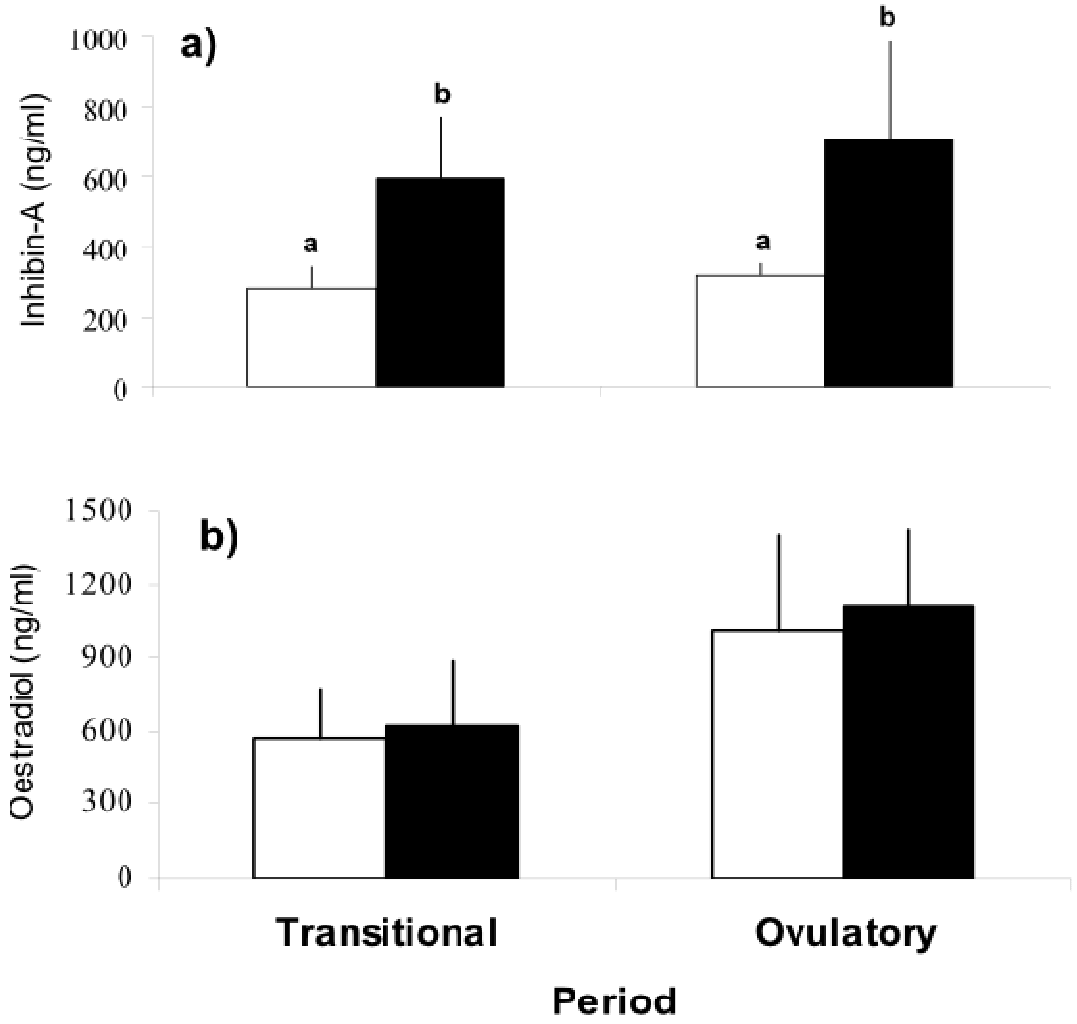
Table 1. Primer sequences used in qPCR analyses of equine granulosa cells

Target	Accession number	Sequences	Product (bp)
IGF1R	X54980.1	For 5'-AAGCTGAGAAGCAGGCAGAG-3' Rev 5'-CGGAGGTTGGAGATGACAGT-3'	265
IGF2R	XM_001491469	For 5'-GTAACGCTAAGCTTTCCTATT ACG-3' Rev 5'–GGTATACCACCGGAAGTTGTA GG-3'	187
FSHR[1]	S70150	For 5'-TCTTTGGCATCAGCACCTAC-3' Rev 5'-AGAAATCCCTGCGGAAGTTC-3'	399
LHCGR	AY464091	For 5'-CCCGGTTAAAATACCTAAGC-3' Rev 5'-AGTGTCGTCCCGTTGAA-3'	229
PAPPA	XM_001487931	For 5'–TCACGCCCAATCAAGT-3' Rev 5'–AGGTCACATGCTGATCC-3'	196
18SRNA	AJ311673	For 5'-GGGGAATCAGGGTTCG-3' Rev 5'-GCTGGCACCAGACTTG-3'	209

[1]Primer sequences from Dell'Aquila et al. (2004)


a)
IGF1R mRNA levels
50
40
30
20
10
0
a
b
*
b)
IGF2R mRNA levels
2
1.5
1
0.5
0
15-24
25-34
c)
FSHR mRNA levels
85
65
45
25
5
a
b
*
d)
LHCGR mRNA levels
20
15
10
5
0
15-24
25-34
e)
PAPPA mRNA levels
2.5
2
1.5
1
0.5
0
15-24
25-34
Diameter range (mm)


a)
Free IGF1 (ng/ml)
6
5
4
3
2
1
0
a
b
a
b
Transitional
Ovulatory
Period
b) IGFBP2
40 Kd
30 Kd
IGFBP2 (arbitrary densitometric units)
1.6
1.2
0.8
0.4
0
Transitional
Ovulatory
Period
c) IGFBP5
30 Kd
20 Kd
IGFBP5 (arbitrary densitometric units)
1.6
1.2
0.8
0.4
0
a
ab
b
a
Transitional
Ovulatory
Period


a)
Inhibin-A (ng/ml)
1000
800
600
400
200
0
a
b
a
b
b)
Oestradiol (ng/ml)
1500
1200
900
600
300
0
Transitional
Ovulatory
Period

APPENDIX 2:

Regulation of the proliferative activity of ovarian surface epithelial cells by follicular fluid.

Regulation of the proliferative activity of ovarian surface epithelial cells by follicular fluid

L.K. Doyle and F.X. Donadeu[1]

The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Roslin BioCentre, Midlothian EH25 9PS, UK

Short title: OSE proliferation and follicular fluid

[1]Correspondence, xavier.donadeu@ed.ac.uk, Tel. (0)131 527 4331, Fax (0)131 440 0434

Abstract

Despite critical roles of the ovarian surface epithelium (OSE) in ovulation and post-ovulatory wound repair, little is known about the physiological mechanism regulating OSE proliferation. A role of follicles and corpora lutea in locally regulating the proliferative activity of OSE has been suggested. In this study, the effects of follicular and luteal products on proliferation of cultured OSE cells were tested using cells obtained from seasonally anoestrous ewes. Follicular fluid but not luteal extracts induced OSE cell proliferation (2.5-fold relative to untreated controls; $P<0.0001$). The response of OSE cells was not affected by follicle size or previous charcoal-extraction of follicular fluid ($P>0.1$). Treatment with IGF-I (2.2 fold; $P<0.01$), EGF (1.9 fold; $P<0.01$) and, to a lesser extent, FSH ($P<0.05$) also induced OSE cell proliferation. In contrast, oestradiol or progesterone did not induce cell proliferation or enhance the effects of FSH on proliferation ($P>0.1$). It was concluded that follicular fluid can directly stimulate ovine OSE cell proliferation and that this effect is attributable to non-steroidal mitogens.

Keywords: Ovarian surface epithelium, cell proliferation, follicular fluid, growth factors, steroids

Introduction

The ovarian surface epithelium (OSE) is composed of a single layer of flat to cuboidal simple epithelial cells that surround the ovary and are separated from underlying stroma by a basement membrane and a dense connective tissue layer, the tunica albuginea (Auersperg *et al.*, 2001). The OSE transports material to and from the peritoneal cavity and plays an important role during ovulation and postovulatory wound repair (reviewed in Auersperg *et al.*, 2001). Most interest on OSE biology has derived from its involvement in ovarian cancer in women. OSE-derived cancers constitute over 90% of all ovarian cancers and are the most aggressive of all gynaecological malignancies (Auersperg *et al.*, 2001).

Despite the extensive literature on the biology of neoplasic OSE (reviewed in Auersperg et al., 2001; Leung and Choi, 2006), relatively little is known on the

physiological mechanisms regulating the proliferation of normal OSE in humans or animals. During normal ovarian cycles the OSE undergoes periodic proliferative changes associated with follicular growth and luteal development (Gaytan *et al.*, 2005). Although regulation of OSE function by systemic factors (e.g., gonadotrophins) in vivo is well established (Stewart et al., 2004; Burdette et al., 2006) recent studies encompassing the entire oestrous cycle in rodents have provided compelling evidence of the importance of local ovarian mechanisms in the regulation of OSE proliferation. Specifically, it was found that cyclic proliferative activity in rat and mouse OSE is localised to areas overlying growing follicles and corpora lutea (Gaytan et al., 2005; Burdette et al., 2006) and this activity is enhanced by gonadotrophins (Davies et al., 1999; Stewart et al., 2004; Burdette et al., 2006).

In addition to gonadotrophins, oestrogens and androgens can stimulate OSE proliferation in *vitro* (Syed et al., 2001; Murdoch and Van Kirk, 2002; Gubbay et al., 2004), although this has not been confirmed in other studies (Karlan et al., 1995; Wright et al., 2002). Instead, progesterone has been reported to block OSE proliferation in vitro in some studies (Syed et al., 2001; Murdoch and Van Kirk, 2002) and this is consistent with a correlation between high levels of progesterone and a reduced risk of ovarian cancer in humans (Auersperg *et al.*, 2001). Several peptide growth factors produced locally in the ovary can also modulate OSE proliferation (Wong and Leung, 2007). Overall, these findings suggest important roles for follicle- and/or luteal-derived products in the regulation of OSE function and perhaps also tumourogenesis.

Rodent models have previously been used to study the biology of normal OSE (Tan and Fleming, 2004; Gaytan et al., 2005; Burdette et al., 2006), however, ruminant models with reproductive cycles more closely resembling humans may be preferable (Parrott et al., 2001; Murdoch and McDonnel, 2002). In addition, ovine OSE have been shown to be an adequate model for in vitro studies on human OSE (Gubbay *et al.*, 2004).

The aim of the present study was to clarify the role of local ovarian regulation of OSE proliferation by determining the effects of follicular and luteal products on the proliferation of ovine OSE cells in culture. The identities of specific follicular/luteal components responsible for such effects were then investigated.

Material and Methods

Cell culture

Pools of surface epithelial cells were obtained from four seasonally anestrous sheep immediatedly after sacrifice. OSE from such animals is expected to be relatively quiescent as it is not subjected to proliferative stimuli associated with periodic postovulatory wound repair and therefore provides an optimum model for testing the effects of substances on OSE cell proliferation. The ovarian surface was gently scraped with the blunt end of a sterile scalpel blade and then rinsed into culture media consisting of M199/MCDB 105 medium (1:1) containing 1 mM L-glutamine, 100UI/ml Penicillin, 100ug/ml Streptomycin and 10% fetal calf serum (FCS), as described (Gubbay *et al.*, 2006). The presence of OSE flakes in the scrapings was confirmed by phase-contrast microscopy. OSE cells from each animal were transferred to separate culture flasks that had been pre-coated with FCS and were incubated in culture media at 37º C in a humidified atmosphere of 5% CO_2. Media was replaced every 7 days until cultures reached confluence (normally after 3 to 4 weeks). Highly pure ovine OSE cultures are consistently obtained using these procedures (Gubbay *et al.*, 2006). Cells were passaged once and used for proliferation assays when they reached confluence.

Cell proliferation assay

Cell cultures derived from different animals were pooled and cells were incubated with culture media in 96-well plates at a density of 5000 cells/well for 24 h. Media was then replaced with media without serum for a further 24 h after which treatments were applied to triplicate wells. These consisted of follicular fluid (final concentration in culture media, 10%) from follicles of different sizes (indicated below) that was either pure or had been charcoal-extracted previously, extracts from mature corpora lutea (final concentration, 10%), recombinant human IGF-I (100 ng/ml; NHPP, Torrance, California), recombinant human EGF (100 ng/ml; Sigma-Aldrich, Dorset, UK), progesterone (3, 30 and 300 ng/ml; Sigma-Aldrich), 17β-oestradiol (3, 30 and 300 ng/ml, Sigma-Aldrich), ovine FSH (100 ng/ml; NHPP), FCS (as supplied by manufacturer, final concentration, 10%, Sigma-Aldrich) or

combinations of these treatments. Cell proliferation was measured with the Cell Titer 96® Aqueous One Solution Proliferation Assay (Promega, Southampton, UK) following instructions by the manufacturer after a 3-day incubation with treatments. According to the manufacturer, this procedure is based on the reduction of an MTS tetrazolium compound into a coloured formazan product by metabolically active cells and the quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture. Proper validation of this method in ovine OSE cells was performed over a range of cell densities (2700 to 135000 cells/well of 96-well plates). Over this range, a linear relationship was found between cell density and optical density (OD) of the samples according to the equation, $OD = (\text{cell density}/10^5) + 0.0343$. ODs for all experimental samples fell well within this linear range.

Follicular fluid used to treat OSE cells was prepared by pooling aspirates from small (2-4 mm), medium (6-8 mm) and large (10-12 mm) follicles taken from six bovine ovaries collected at an abattoir. Preliminary data (not shown) demonstrated that OSE had similar proliferative responses to bovine and ovine follicular fluid. A fraction from each bovine follicular fluid pool was charcoal-extracted to remove steroids and other lipids as described (van Tol and Bevers, 2001). Follicular fluid was filter-sterilised and frozen at -20C for later use. Oestradiol concentrations in follicular fluid were measured by RIA (England *et al.*, 1981) and they were found to be, before charcoal-extraction, 8.9, 69.9 and 594.3 ng/ml in fluid from small, medium and large follicles, respectively. Oestradiol levels were reduced by charcoal-extraction to 1.3, 0.8 and 8.2 ng/ml in the three types of fluid, respectively. Luteal extracts were obtained by grinding four freshly collected, mature corpora lutea from bovine ovaries followed by filtration, centrifugation, collection of supernatants into OSE culture media and filter-sterilisation before storage at -20C.

Statistical analyses

In each case, the mean values from triplicate wells were used for statistical analyses. Data were analyzed by one-way ANOVA using the General Linear Model (Minitab 15). When a main effect was significant or approached significance,

Tukey's test was used to locate differences between means. Main effects and differences between means were considered to be significant when $P \leq 0.05$.

Results

Incubation of cultured ovine OSE cells with follicular fluid resulted in a 2.5-fold increase in cell proliferation relative to untreated controls ($P<0.0001$), which was approximately 40% the proliferative response induced by FCS (Figure 1A). There were no effects ($P>0.1$) of follicle size or previous charcoal extraction in the proliferative responses to follicular fluid. Luteal extracts, in contrast, did not stimulate cell proliferation ($P>0.1$). IGF-I or EGF induced proliferation of OSE cells (means of 2.2- and 1.9-fold over untreated controls, respectively; $P<0.01$; Figure 1B). In contrast, progesterone or oestradiol had no effects on cell proliferation at any of the doses used ($P>0.1$). FSH had a small but significant effect on cell proliferation (mean, 1.2-fold; $P<0.05$). Proliferative responses to FSH in the presence of oestradiol or progesterone were not significantly different ($P>0.1$) to those of control cells or cells treated with FSH alone.

Discussion

Cyclic proliferative activity in OSE has been reported to be localised to areas overlying growing follicles and corpora lutea (Gaytan et al., 2005; Burdette et al., 2006), suggesting a stimulatory effect (direct or indirect) of follicular and/or luteal products on OSE proliferation. The effects of some of these products, particularly steroids, on OSE cell proliferation in culture have been studied, although results have been inconclusive (Karlan et al., 1995; Syed et al., 2001; Murdoch and Van Kirk, 2002; Wright et al., 2002; Stewart et al., 2004). Therefore, we decided to test the direct effects of whole follicular fluid and luteal extracts on OSE cell proliferation and then investigate individual components that may account for any observed effects. Our results show for the first time that follicular fluid but not luteal extracts stimulate proliferation of ovine OSE in culture. The observed effects of follicular fluid on OSE proliferation and the smaller response of OSE to follicular fluid than to serum were not surprising since follicular fluid is partially an exudate of serum and therefore it is expected to contain at least some of the mitogenic factors found in

serum (Murray *et al.*, 1998). In addition, the levels of some of these mitogens, such as IGF-I, are higher in serum than in follicular fluid (Kirby et al., 1993). Follicular fluid has been shown to induce the proliferation of other non-follicular cell types such as endometrial and endothelial cells (Bahtiyar et al., 1998; von Otte et al., 2006).

The absence of an effect of charcoal extraction on the proliferative responses of OSE cells to follicular fluid and the inability of oestradiol or progesterone, at any of the three doses chosen to encompass physiological levels in ovine follicular fluid (Spicer et al., 1995; Evans et al., 2000), to induce significant cell proliferation or to modify the response of OSE cells to FSH excludes a steroidal component being responsible for the mitogenic effects of follicular fluid on OSE cells. Several non-steroidal growth factors have been shown to stimulate OSE proliferation in vitro, including members of the IGF and EGF families and gonadotrophins (Gubbay et al., 2004; Wong and Leung, 2007), specific receptors for which are expressed in OSE (Auersperg et al., 2001). In the present study, IGF-I and EGF induced, on average, 2.2- and 1.9-fold increases in OSE proliferation, respectively, which are similar to the responses induced by follicular fluid (combined average for all follicle sizes, 2.3-fold increase). The dose of IGF-I used in this study was within the range of concentrations reported in follicular fluid (Kirby et al., 1993; Artini et al., 1994). In contrast, the dose of EGF in follicular fluid has been reported to be substantially lower to that of IGF-I (Artini et al., 1994). Therefore, although the two growth factors had similar effects on OSE proliferation in this study, their relative roles in vivo may be different. The effect of FSH on proliferation, albeit relatively low, was consistent with previous results in vitro (Gubbay *et al.*, 2006) and in vivo (Stewart et al., 2004; Burdette et al., 2006). Taken together, these findings suggest that the mitogenic effects of follicular fluid on OSE derive, to a considerable extent, from IGF-I and EGF, and possibly other mitogenic peptides (Khan et al., 1997; Wong and Leung, 2007), rather than from direct effects of follicular steroids. This finding should be confirmed in future studies by assessing the effects of follicular fluid depletion of IGF-I and EGF on proliferative responses of OSE.

The present findings imply potentially important role(s) of follicular fluid in OSE remodelling during the oestrous/menstrual cycle. Such role(s) would most readily

take place after the release of the follicular contents into the abdominal cavity and over the ovarian surface during ovulation as this may provide a strong stimulus for OSE proliferation thus contributing to initiate the re-epithelisation of the ovulatory wound. Proliferative responses in this study resulted from a 3-day exposure of cultured OSE to follicular fluid. OSE responses to follicular fluid after ovulation in vivo would presumably follow different kinetics, due for example to relative shorter exposure of OSE cells to mitogenic stimuli, and therefore the magnitude of such response would be different from that observed in vitro. Further studies will be needed to address this.

In contrast to the direct effects on the ovulatory wound, a mitogenic effect of follicular fluid on the OSE overlying developing follicles would depend on the ability of mitogenic products to diffuse from the follicular fluid compartment to the OSE compartment. Such an influence of follicular fluid may readily occur during the latest stages of maturation of the ovulatory follicle when considerable thinning of the follicle wall and adjacent tunica albuginea occur in preparation for ovulation (Murdoch and McDonnel, 2002), but may have a less important role during earlier stages of follicular growth.

In this study, in contrast to follicular fluid, CL extracts did not induce significant proliferative activity. Results from a preliminary study (not showed) demonstrated that these differences were not due to differences in protein content between follicular fluid and CL extract. A possible explanation for the present result is that mitogenic substances produced in the highly vascularised corpus luteum may not accumulate locally since they would be continually cleared into the circulation. Luteal stimuli, therefore, may not play a significant role in the re-epithelisation of the ovulatory wound. Instead, this process may largely involve stimulatory signals generated locally at the borders of the ovulatory wound and adjacent stroma in response to ovulation-induced inflammation and rupture of OSE integrity, similar to the mechanisms involved in regeneration of other body epithelia (Grinnell, 1992). This conclusion is consistent with responses to experimentally induced OSE injury (Stewart et al., 2004; Wright et al., 2007). An alternative explanation for the lack of effects of CL extracts on proliferation is that these extracts as obtained in the present

study may not contain the full mitogenic potential of the CL, a possibility that cannot be ruled out.

In summary, follicular fluid but not luteal extracts stimulated proliferation of cultured ovine OSE cells. The effects of follicular fluid were not dependent on follicle size and were attributed, at least partially, to IGF-I and EGF but not to follicular steroids. Follicular fluid from ovulatory follicles may have an important role in remodelling of the ovarian surface by promoting OSE proliferation during the peri-ovulatory period.

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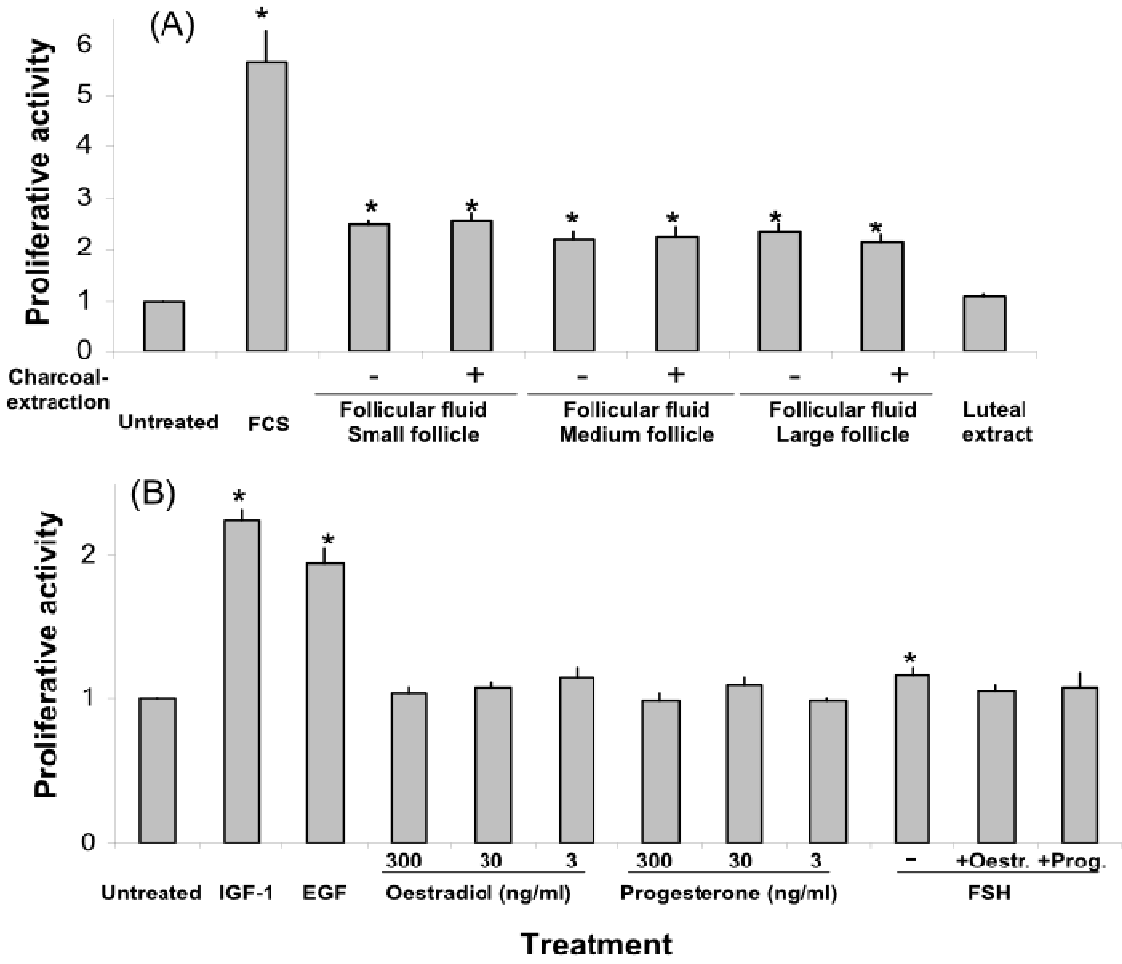
Figure legends

Figure 1. Proliferative responses (mean ± SEM; n=4 experiments) of cultured ovine OSE cells to (A) FCS, follicular fluid and luteal extracts and (B) IGF-I, EGF, oestradiol, progesterone and FSH (alone or in combination with oestradiol or progesterone). Follicular fluid was separately pooled from small (2-4 mm), medium (6-8 mm) or large (10-12 mm) bovine follicles and a fraction from each pool was charcoal-extracted. Values for each treatment are expressed relative to the value in untreated cells which was taken as 1. In each case, stars indicate treatment means that are different from the mean value in untreated controls ($P<0.05$).

Figure


(A)
Proliferative activity
0
1
2
3
4
5
6
Charcoal-extraction
-
+
-
+
-
+
Untreated
FCS
Follicular fluid Small follicle
Follicular fluid Medium follicle
Follicular fluid Large follicle
Luteal extract
(B)
Proliferative activity
0
1
2
300
30
3
300
30
3
-
+Oestr.
+Prog.
Untreated
IGF-1
EGF
Oestradiol (ng/ml)
Progesterone (ng/ml)
FSH
Treatment